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TAHIRA ANWAR

AUTOPHAGOSOME BIOGENESIS: ATG4, TRIM17 AND BECLIN 1 LOCALIZATION

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University of Helsinki, Finland

Autophagosome Biogenesis: ATG4, TRIM17 and Beclin 1 localization

Tahira Anwar

ACADEMIC DISSERTATION

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To know what is right and not to do it is the worst cowardice

(Confucius)

“All hope abandon, ye who enter here”

(The Divine Comedy, Dante Alighieri)

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LIST OF ORIGINAL PUBLICATIONS

I Mandell MA, Jain A, Kumar S, Castleman MJ, **Anwar T**, Eskelinen EL, Johansen T, Prekeris R, Deretic V. TRIM17 contributes to autophagy of midbodies while actively sparing other targets from degradation. Journal of Cell Science. 2016 Oct 1;129(19):3562-3573.

II Syrjä P, **Anwar T**, Jokinen T, Kyöstilä K, Jäderlund KH, Cozzi F, Rohdin C, Hahn K, Wohlsein P, Baumgärtner W, Henke D, Oevermann A, Sukura A, Leeb T, Lohi H, Eskelinen EL. Basal autophagy is altered in Lagotto Romagnolo dogs with an ATG4D mutation. Veterinary Pathology. 2017 Nov;54(6):953-963.

III **Anwar T**, Liu X, Suntio T, Marjamäki A, Biazik J, Chan EYW, Varjosalo M, Eskelinen EL. ER-targeted Beclin 1 supports autophagosome biogenesis in the absence of ULK1 and ULK2 kinases. Cells. 2019 May 17;8(5).

The publications are referred to in the text by their roman numbers (I-III).
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The author´s contribution to the publications:

I Performed the experimental work and interpretation of results from correlative light electron microscopy.

II Performed the planning, experimental work and interpretation of results from the experiments including western blotting. Contributed to the immunofluorescence image analysis and editing of the manuscript.

III Designed the experimental study, performed the experimental work except for mass spectrometry, performed data analysis and wrote the manuscript.

ABBREVIATIONS

ALPS	Amphipathic lipid-packing sensor
ALR	Autophagic lysosome reformation
AMPK	Adenosine monophosphate-activated protein kinase
ATG	Autophagy-related
BATS	Barkor/ATG14(L) autophagosome targeting sequence
BH3	Bcl-2 homology 3 domain
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
CCD	Coiled-coil domain
CR	Caloric restriction
DFCP1	Double-FYVE-containing protein 1
DAPK	Death-associated protein kinase
EAT	Early autophagy targeting and tethering domain
ECD	Evolutionarily conserved domain
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FIP200	Focal adhesion kinase family interacting protein of 200 kDa
GABARAP	γ-Aminobutyric acid receptor-associated protein
HEK	Human embryonic kidney cell line
HeLa	Human cervical carcinoma cell line (Henrietta Lacks)
KIR	Keap1-interacting region
LAMP2A	Lysosome-associated membrane protein 2A
LAPTM4B	Lysosomal protein transmembrane 4B
LC3	Microtubule-associated protein 1A/1B-light chain 3
LIR	LC3 interacting region
LR	Lagotto Romagnolo
MEF	Mouse embryonic fibroblasts
MAPK	Mitogen-activated protein kinase
MEK/ERK	MAPK/extracellular signal–regulated kinase / extracellular signal-regulated kinase
MST4	Mammalian STE20-like protein kinase 4
mTORC1	Mechanistic target of rapamycin complex 1
MVB	Multivesicular body
NES	Nuclear export signal
NLRP3	NLR family pyrin domain-containing protein 3
NRK	Normal rat kidney cells
OPTN	Optineurin
PDCD6IP	Programmed cell death 6 interacting protein
PE	Phosphatidylethanolamine

PI3P	Phosphatidylinositol-3-phosphate
PP2A	Protein phosphatase 2A
RING	Really interesting new gene domain
Rubicon	Run domain Beclin-1-interacting and cysteine-rich domain-containing protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNAP	Synaptosomal nerve-associated protein
SNAREs	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SQSTM1	Sequestosome 1
STX17	Syntaxin 17
TAX1BP1	Tax1-binding protein 1
TMRE	Tetramethylrhodamine, ethyl ester
TRIM	Tripartite motif
UBD	Ubiquitin-binding domains
ULK	Unc-51-like-kinase
UVRAG	UV radiation resistance-associated gene protein
VAMP	Vesicle-associated membrane protein
VPS	Vacuolar protein sorting
WIPI	WD-repeat domain phosphoinositide-interacting proteins

ABSTRACT

Autophagy is an evolutionarily conserved pathway used by cells to degrade cellular cargoes that are larger than individual proteins in lysosomes. Autophagy maintains cellular homeostasis, provides building blocks for energy production, degrades aggregate-prone proteins and/or whole organelles and fights against invading pathogens. Impaired autophagy plays a significant role in human diseases such as neurodegeneration, cancer and infections. During autophagy, a double-membrane structure, named phagophore, forms around the cytoplasmic cargo. The phagophore grows and seals in order to form a double-membrane vesicle named autophagosome. The autophagosome then fuses with a lysosome to gain degradative properties, forming a structure called the autolysosome. Within the autolysosome, the sequestered cargo is degraded, and the resulting building blocks are recycled back to the cytoplasm for energy production or biosynthetic reactions.

Autophagosome biogenesis is tightly regulated by the different components of the autophagy machinery encoded by the autophagy-related (ATG) genes. Beclin plays a key role in autophagy initiation. The importance of Beclin 1 relies on its binding to the phosphatidylinositol 3-kinase VPS34. The Beclin 1/VPS34 complex produces phosphatidylinositol 3-phosphate (PI3P) which is necessary for the recruitment of downstream autophagy effectors involved in autophagosome biogenesis. Autophagy is severely impaired in the absence of VPS34 kinase activity. The tripartite motif (TRIM) family proteins play an important role in selective autophagy. They bind to cellular degradative cargo acting as autophagy receptors. In addition to this, they regulate autophagy initiation by acting as a platform for the assembly of the Beclin 1 and ULK complexes. TRIM17 inhibits bulk autophagic degradation of various cellular targets, unlike most TRIMs, which have positive roles. Two ubiquitin-like conjugation systems, the ATG12-ATG5 system and the LC3-phospha-tidylethanolamine (LC3-PE) system are required for phagophore expansion. The cysteine protease ATG4 is a key player in LC3-PE conjugation. ATG4 cleaves pro-LC3 to expose a glycine residue at the C-terminus, to which the PE is then conjugated. Upon autophagosome closure, ATG4 delipidates LC3 from the outer autophagosomal membrane releasing it back to the cytoplasm for the next round of LC3-PE conjugation.

Many aspects of the molecular mechanisms regulating autophagosome biogenesis in starvation-induced and selective autophagy are still unresolved. Moreover, the origin of the phagophore still holds many unanswered questions. Beclin 1, TRIM17 and ATG4 are all important checkpoints in autophagosome biogenesis. Autophagy is connected to several human diseases. To date, there are no effective therapies for the prevention or treatment of diseases that specifically target autophagy. A better

understanding of the molecular mechanisms regulating autophagy could potentially lead to novel clinical applications towards effective disease treatments.

In collaboration with the research group of Prof. Vojo Deretic, we studied the role of the protein TRIM17 in selective autophagy and found that TRIM17 inhibits selective autophagy of various known targets while targeting midbodies for autophagic degradation. The protein Mcl-1 regulates autophagy-inducing or -inhibiting functions of TRIM17 in the cell. The formation of a TRIM17-Beclin 1-Mcl-1 complex inhibits selective autophagy. TRIM17-dependent selective degradation of midbodies via autophagy is induced upon dissociation of Mcl-1 from the Beclin 1-TRIM17 complex.

In collaboration with Pernilla Syrjä, we revealed that Lagotto Romagnolo dogs carrying an ATG4D missense mutation exhibit altered basal autophagy and abnormal cytoplasmic vacuolization. Moreover, our study confirmed normal lysosomal degradation in affected dogs thus excluding a link between the ATG4D mutation and lysosomal storage diseases.

Finally, we showed that, upon starvation, Beclin 1 targeted to the endoplasmic reticulum (ER) partially rescued autophagosome formation in cells deficient in ULK1 and ULK2 kinases. The autophagy flux was impaired in cells lacking ULK1 and ULK2 kinases and this defect was not rescued by expression of Beclin 1 targeted to the ER. These results suggest a regulatory role for the ULK kinases in autophagosome maturation in addition to autophagosome biogenesis. Moreover, our study demonstrated that ULK1 and ULK2 kinases might play a role in regulating Beclin 1 enrichment in the ER under nutrient-rich conditions.

1 INTRODUCTION

1.1 Autophagy

In autophagy, cytoplasmic material is transported to lysosomes for degradation and recycling. Along with the ubiquitin-proteasome system (UPS), responsible for intracellular quality-control, autophagy is a major catabolic pathway that delivers non-functional cellular cargo to lysosomes for degradation. It is conserved from yeast to mammals, important for cellular homeostasis and allows degradation of protein aggregates as well as unused or damaged organelles. Cargo degradation releases “building blocks” back to the cell to be recycled for biosynthesis and energy production. Defective autophagy plays a role in several diseases including neurodegenerative, muscular and infectious diseases as well as cancer (Suzuki and Ohsumi 2010, Parzych and Klionsky 2014, Bento et al. 2016, Dikic and Elazar 2018). There are three main types of autophagy, namely macroautophagy (simply referred to as autophagy), microautophagy and chaperone-mediated autophagy (CMA) (Galluzzi et al. 2017) (Figure 1).

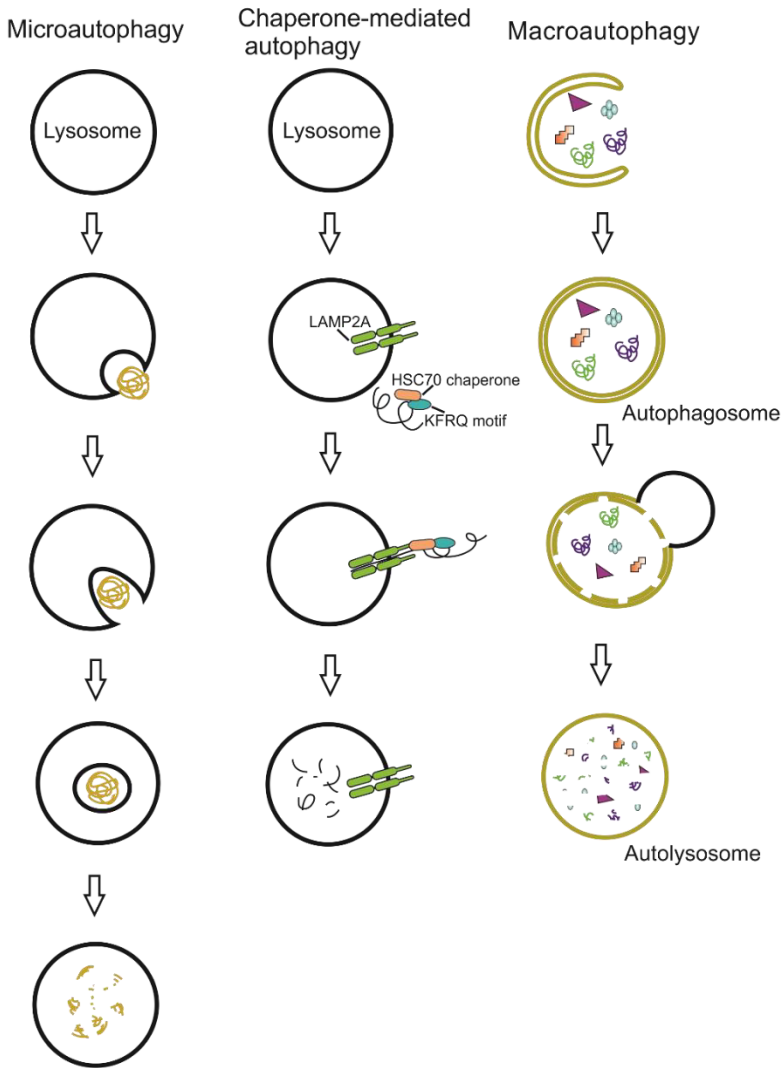

***Figure 1**: Schematic representation of the three autophagic pathways: microautophagy, chaperone-mediated autophagy and macroautophagy (see text for details).*

In microautophagy, the cytoplasmic cargo directly enters the lysosome or endosome by inward budding of the organelle limiting membrane. The content is then degraded by lysosomal hydrolases (Tekirdag and Cuervo 2018). Chaperone-mediated autophagy, a highly specific process, requires the activity of cytoplasmic and lysosomal chaperones (Kaushik and Cuervo 2018). All CMA substrates contain a

peptide signal similar to the KFERQ sequence that is recognized during CMA by the cytosolic chaperone HCS70. The chaperone-bound cargo is then delivered to the lysosome assisted by the lysosome-associated membrane protein 2A (LAMP2A). Here, the cargo is unfolded and internalized through the lysosomal membrane for final degradation. Macroautophagy differs from microautophagy and CMA as the initial sequestration of the cytoplasmic cargo requires the formation of a separate vesicle called the autophagosome (Mercer et al. 2018). Macroautophagy starts with the formation of a phagophore, also known as isolation membrane, that surrounds the cellular degradative cargo consisting of components such as long-lived proteins, organelles or invasive pathogens (Figure 2).

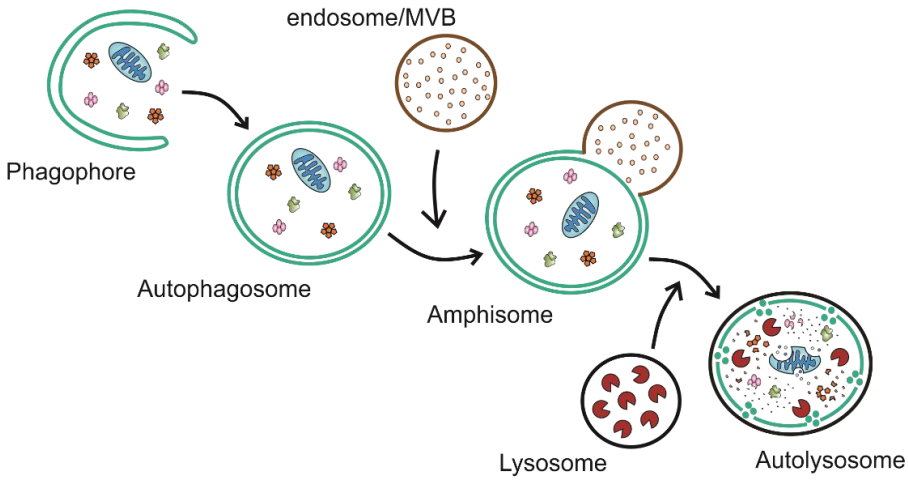

***Figure 2**: Schematic representation of the macroautophagic pathway. Cytoplasmic cargo is sequestered by a flat membrane cisterna called the phagophore (or isolation membrane). The phagophore closes to form a double-membrane vesicle called the autophagosome. This then fuses with endosomes and lysosomes forming an autolysosome where the cargo is degraded and recycled back to the cytoplasm.*

The isolation membrane expands until forming the double-membrane vesicle called the autophagosome containing the cargo. The autophagosome then fuses with endosomes forming an intermediate vesicle called the amphisome. Finally, the amphisome fuses with a lysosome forming a structure called the autolysosome. Here, the outer autophagosome membrane fuses with the lysosomal membrane, while the

inner membrane is degraded together with the cargo itself by lysosomal proteases. The degraded content is recycled back to the cytoplasm for reuse (Parzych and Klionsky 2014).

1.2 Autophagy machinery and regulation

The discovery of the autophagy-related (ATG) genes in *Saccharomyces cerevisiae* provided a considerable breakthrough in deciphering the molecular machinery regulating autophagy (Tsukada and Ohsumi 1993, Thumm et al. 1994, Harding et al. 1995). To date, around 33 genes have been identified and most of them are conserved from yeast to mammals (Mizushima et al. 2011). The Atg proteins involved in autophagy are divided into six functional units: the Atg1 (yeast)/Unc-51-like kinase 1 and 2 (ULK1/2, mammals) and its regulators; the class III phosphatidyl inositol 3-kinase (PI3K) complex, or Atg6 (yeast)/Beclin 1 (mammals)/Vps34-Atg14 complex; the transmembrane protein Atg9/ATG9; the Atg2 and Atg18/WIPI1-4 PI3P-binding proteins; the Atg12-ATG12/Atg5-ATG5 conjugation system and the Atg8 (yeast) /LC3 (mammals)-phosphatidylethanolamine conjugation system (Table 1, Figure 3). Mammals have several ATG8 homologs including LC3s and GABARAPs.

Table 1: *Autophagic proteins in yeast and mammals*

Functional unit	Yeast	Mammals	Function
Atg1/ULK complex	Atg1	ULK1 and ULK2	Ser/Thr kinase; autophagy initiation
	Atg11	Unknown	Scaffolding protein
	Atg13	ATG13	Scaffolding protein
	Atg17	Unknown	Scaffolding protein
	Atg29	Unknown	Atg1 complex assembly
	Atg31	Unknown	Atg1 complex assembly
	Unknown	FIP200/RB1CC1	Scaffolding protein
	Unknown	ATG101	Stabilizes ATG13 and ULK1
Class III PI3K complex	Atg6/Vps30	Beclin 1	Scaffolding protein; autophagy initiation
	Vps34	VPS34	PI3K; produces PI3P

Table 1: *Autophagic proteins in yeast and mammals (continued)*

Functional unit	Yeast	Mammals	Function
Class III PI3K complex	Vps15	VPS15	Ser/Thr kinase; VPS34 regulatory subunit
	Atg14	ATG14	Targets complex to lipid membranes
Other proteins	Atg2	ATG2	Autophagosome closure and lipid droplets distribution, putative lipid transfer protein
	Atg9	ATG9	Membrane shuttling
	Atg18	WIPI1-4	PI3P binding proteins
	Unknown	DFCP1	PI3P binding protein; localizes to omegasomes
Atg12/ATG12 conjugation system	Atg5	ATG5	Autophagosome elongation
	Atg7	ATG7	E1-like enzyme; activates Atg8/ATG8s and Atg12/ATG12
	Atg10	ATG10	E2-like enzyme; conjugates Atg12/ATG12 to Atg5/ATG5
	Atg12	ATG12	Ubiquitin-like protein
	Atg16	ATG16	Autophagosome elongation

Table 1: *Autophagic proteins in yeast and mammals (continued)*

Functional unit	Yeast	Mammals	Function
Atg8/ATG8 conjugation system	Atg3	ATG3	E2-like enzyme; catalyses lipid conjugation
	Atg4	ATG4A-D	Cysteine protease involved in Atg8/ATG8 lipidation and delipidation
	Atg7	ATG7	E1-like enzyme; activates Atg8/ATG8 and Atg12/ATG12
	Atg8	LC3A-C; GABARAP, GABARAPL1-3	Autophagosome marker; ubiquitin-like protein

Abbreviations: DFCP1, double FYVE-containing protein 1; GABARAP, γ-aminobutyric acid receptor-associated protein; LC3, microtubule-associated protein 1A/1B-light chain 3; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; ULK, unc-51-like kinase; VPS, vacuolar sorting protein; WIPI, WD-repeat phosphoinositide-interacting protein.

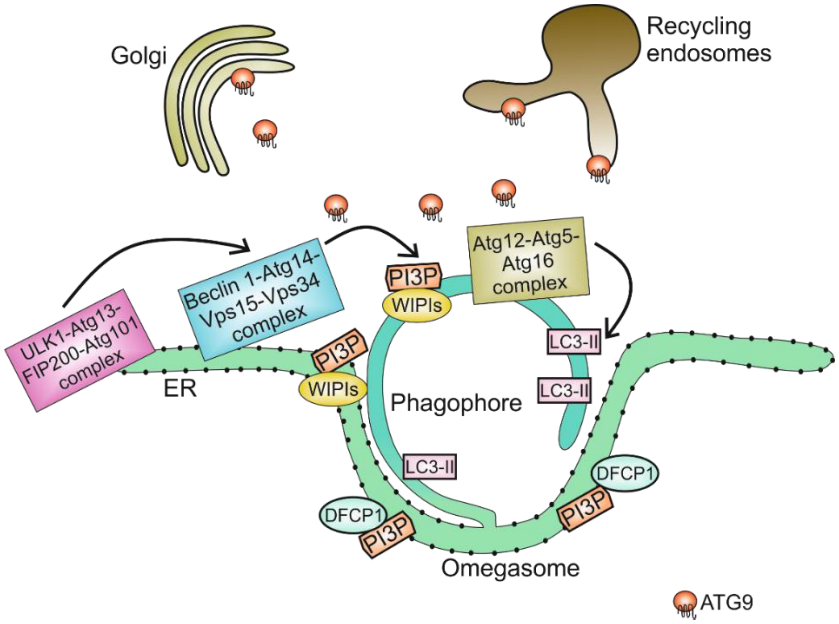

***Figure 3**: Autophagosome formation. The ULK1 and the Beclin 1/VPS34 complexes translocate to the site of autophagosome formation. Production of PI3P recruits DFCP1 and WIPI proteins; the latter then recruits the ATG12-ATG5 complex allowing LC3 lipidation to the forming phagophore. ATG9 vesicles potentially deliver lipid for membrane elongation from various membrane sources.*

1.2.1 Autophagy initiation: ULK1/2 complex and ATG9 vesicles

Atg1, a serine/threonine protein kinase, is highly conserved among eukaryotes, and its mammalian homologs are Unc-51-like kinases 1 and 2 (ULK1 and ULK2) (Mizushima 2010, Grasso et al. 2018, Mercer et al. 2018). The isoform ULK1 plays a major role in autophagy initiation while the role of ULK2 in autophagy is less clear. It has been shown that ULK1-deficient mice present only slight abnormalities suggesting a redundant role for ULK2 (Kundu et al. 2008). Autophagy is severely impaired in the absence of both ULK1 and ULK2 kinases (McAlpine et al. 2013), and ULK1/ULK2 double-deficient mice die shortly after birth (Cheong et al. 2011). ULK deficiency in vivo blocks starvation-induced autophagy and activates a misregulated unfolded protein response (UPR) in neuronal cells (Joo et al. 2016).

The protein ULK1 contains an N-terminal serine/threonine protein kinase domain, a central proline/serine (P/S)-rich domain and a conserved C-terminal domain (CTD).

The ULK1 complex contains ULK1 itself, the scaffolding subunit FIP200 (focal adhesion kinase or FAK) family-interacting protein of 200 kDa, also known as RB1CC1), ATG13 and ATG101 (Mizushima 2010). ULK1 activity in autophagy is regulated by the mechanistic target of rapamycin mTOR complex 1 (mTORC1). mTORC1 is a serine/threonine kinase that coordinates signals from amino acids, oxygen and growth factor levels and controls cell growth (Bar-Peled and Sabatini 2014). In nutrients-rich conditions, mTORC1 phosphorylates ULK1 on Ser757 and ATG13 on Ser258 inhibiting ULK1 complex activity and hindering autophagy induction (Figure 4) (Ganley et al. 2009, Jung et al. 2010, Kim et al. 2011, Puente et al. 2016). Moreover, ULK1 auto-phosphorylates itself and phosphorylates FIP200 under nutrient-rich conditions.

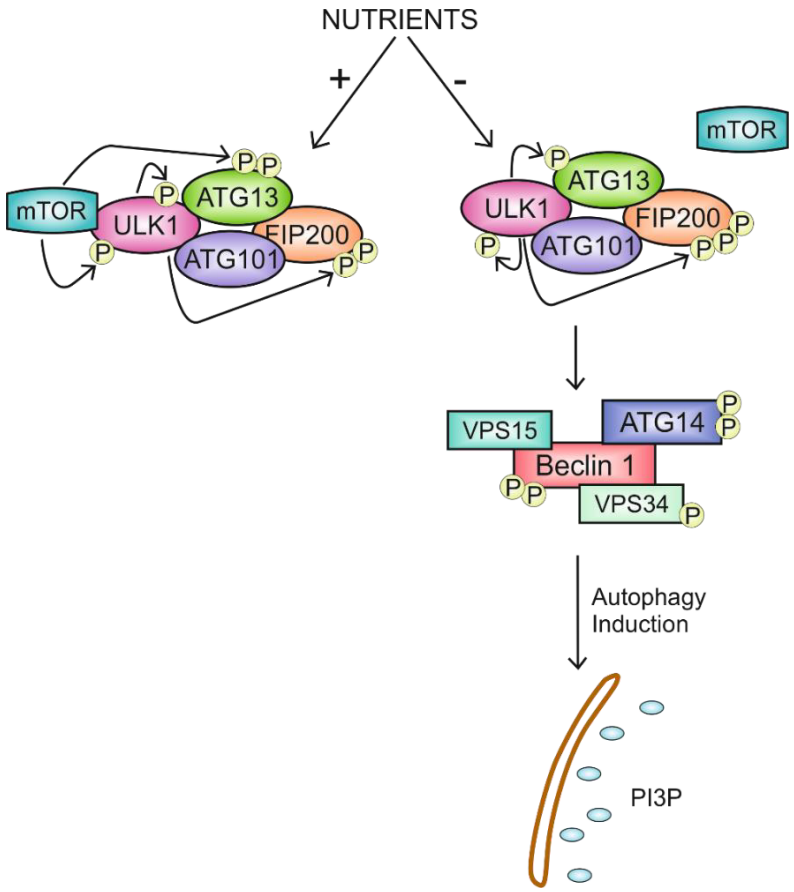

***Figure 4**: Phosphorylation events of the ULK1 complex during autophagy (see text for details).*

Upon starvation, mTORC1 dissociates from the ULK1 complex leaving the inhibitory mTORC1-dependent phosphorylation sites in ULK1 and ATG13 dephosphorylated. Active ULK1 phosphorylates itself and the binding partners Atg13 and FIP200 leading to induction of autophagy. The activated ULK1 complex translocates from the cytoplasm to subdomains of the endoplasmic reticulum (ER) called omegasomes where it drives the nucleation of the autophagosome (Karanasios et al. 2013). The EAT (early autophagy targeting and tethering) domain at the C-terminus of the protein ULK1 facilitates the recruitment and subsequent anchoring of the ULK1 complex to the omegasome (Chan et al. 2009). Moreover, both ULK1 and ATG13 proteins contain a LIR (LC3 interacting region) motif for LC3 binding; this interaction enhances the affinity of the ULK1 complex towards the site of autophagosome initiation (Alemu et al. 2012, Kraft et al. 2012).

Upon activation, ULK1 phosphorylates numerous substrates important for correct autophagosome formation. ULK1 phosphorylates several components of the class III phosphatidylinositol-3 kinase VPS34 (vacuolar sorting protein 34, PI3KC3) complex I (Figure 4). ULK1 also phosphorylates Beclin 1 at Ser15, enhancing the activity of the VPS34 kinase and promoting autophagy (Russell et al. 2013, Egan et al. 2015). ULK1 phosphorylates VPS34 also at Ser249 but the effects of this modification are still unclear (Egan et al. 2015). Lastly, ULK1 phosphorylates also ATG14 at Ser29, activating the PI3KC3 complex and inducing autophagosome initiation (Park et al. 2016). The protein AMBRA1, important for autophagosome formation, is also a substrate of ULK1 phosphorylation (Di Bartolomeo et al. 2010). The Beclin 1/VPS34 complex is sequestered at the cytoskeleton via Beclin 1-AMBRA1 interaction with the dynein light chains 1/2. Upon autophagy induction, ULK1 phosphorylates AMBRA1, releasing the Beclin1/VPS34 complex from the dynein motors and allowing its relocation to the site of autophagosome formation.

Ubiquitination of the protein ULK1 also plays a role in autophagy regulation via the ULK1 complex. Upon autophagy induction, AMBRA1 interacts with TRAFF6 (tumor necrosis factor receptor (TNFR)-associated factor 6) which ubiquitinates ULK1 at Lys63, promoting its self-association and activation (Nazio et al. 2013). Moreover, upon starvation, NEDD4L (neural-precursor-cell-expressed develop-mentally down-regulated 4L) and CULLIN3 negatively regulate autophagy by targeting ULK1 for proteasomal degradation through ubiquitination of Lys27 and Lys29 (NEDD4L) and Lys48 (CULLIN3) (Liu et al. 2016, Nazio et al. 2016).

In parallel with mTORC1, ULK 1 is also regulated by the AMP-activated protein kinase (AMPK). AMPK phosphorylates ULK1 on Ser317 and Ser777, activating the ULK1 complex and inducing autophagy (Kim et al. 2011).

ATG9 is a multi-spanning membrane protein containing six highly conserved transmembrane domains (Young et al. 2006). ATG9 is localized in the Golgi complex and in early, late and recycling endosomes under nutrient-rich conditions. It is thought that ATG9 delivers membranes to the forming autophagosome by shuttling between the phagophore formation site and the Golgi complex, endosomes or other membrane sources (Orsi et al. 2012). ATG9 shuttling is dependent on ULK1 kinase activity. ULK1 phosphorylates ATG9 at Ser14, regulating its cellular dynamics as well as autophagy initiation (Zhou et al. 2017). Knockdown of ULK1 blocks the shuttling of ATG9 from the trans-Golgi network to the phagophore formation site (Orsi et al. 2012).

1.2.2 Autophagy initiation: Beclin 1 protein and Beclin 1 complexes

Beclin 1, the mammalian ortholog of yeast Atg6/Vps30, is the core component of the VPS34 lipid kinase complex (Kihara et al. 2001). The Beclin1/VPS34 complex acts immediately downstream of the ULK1 complex in autophagy initiation. The BECN1 gene is essential for embryonic survival and development and it is an oncosuppressor found to be monoallelically deleted in various cancers (Liang et al. 1999, Yue et al. 2003).

Beclin 1, first discovered as a Bcl-2-interacting protein, is a 450 amino acids long protein in humans (480 amino acids in mouse). Beclin 1 acts as a platform for the assembly of the class III PI3K complex I via protein-protein interactions involving Beclin 1 protein domains (Figure 5) (Oberstein et al. 2007, Huang et al. 2012).

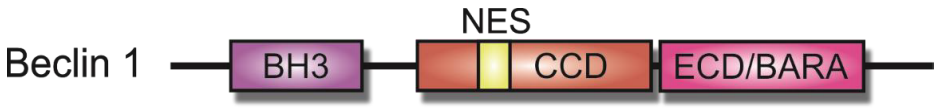

Figure 5*: Schematic representation of Beclin 1 protein domain structure. BH3 (Bcl-2 homology 3); CCD (coiled-coil domain); ECD (evolutionarily conserved domain); NES (nuclear export signal); BARA (β-α repeated, autophagy-related).*

The N-terminal BH3 (Bcl-2 homology 3) domain binds to Bcl-2 family proteins, and this interaction inhibits autophagy. Autophagy is regulated by proteins other than Bcl-2 that interact with Beclin 1 via the BH3 domain. For example, VMP1, vacuole membrane protein 1, interacts with Beclin 1 through the BH3 domain, releasing it from

Bcl-2 and allowing its interaction with VPS34 and consequent autophagy induction (Molejon et al. 2013).

The CCD (coiled-coil domain) is required for hydrophobic protein-protein interactions. Self-association of Beclin 1 proteins takes place via the CCD (Matsunaga et al. 2009). Beclin 1 homodimers are metastable, and the CCD interface permits the separation of the Beclin 1 dimers allowing formation of the more stable Beclin 1-ATG14 and Beclin 1-UVRAG (UV radiation resistance-associated gene protein) heterodimers. Beclin 1 inactive homodimers form a pool of Beclin 1 proteins available for assembly of new Beclin 1-VPS34 complexes. It has been shown that the structure of Beclin 1 CCD contains polar or charged residues in the interface of Beclin 1 dimers causing the homodimers to be unstable, promoting more stable interactions between Beclin 1 and ATG14 or UVRAG (Li et al. 2012). Despite this, it is still unclear how ATG14 and UVRAG employ these residues to form stable interactions. Within the CCD, there is also a leucine-rich nuclear export signal motif important for Beclin 1 translocation from the nucleus to the cytoplasm (Liang et al. 2001). Mutations of this export signal motif result in Beclin 1 accumulation in nuclei, causing impaired autophagy and blocking the tumor-suppressor activity of Beclin 1.

The C-terminus of the protein Beclin 1 contains the ECD (evolutionarily conserved domain). Structural studies of the C-terminus of Beclin 1 identified a novel class of membrane-binding domain, called BARA (β-α repeated, autophagy-related) (Huang et al. 2012). This domain comprises three internal repeats, and each of them includes a pair of β-strands followed by an α-helix. A hydrophobic protrusion in the BARA domain, consisting of three aromatic residues, permits Beclin 1 binding to lipid membranes enriched in cardiolipin. Mutations of the aromatic finger hinder omegasome formation and autophagy initiation (Huang et al. 2012).

Beclin 1 and VPS34 form different complexes that are involved in the regulation of autophagosome biogenesis, autophagosome maturation and endocytic trafficking (Figure 6) (Matsunaga et al. 2009, Zhong et al. 2009, Thoresen et al. 2010).

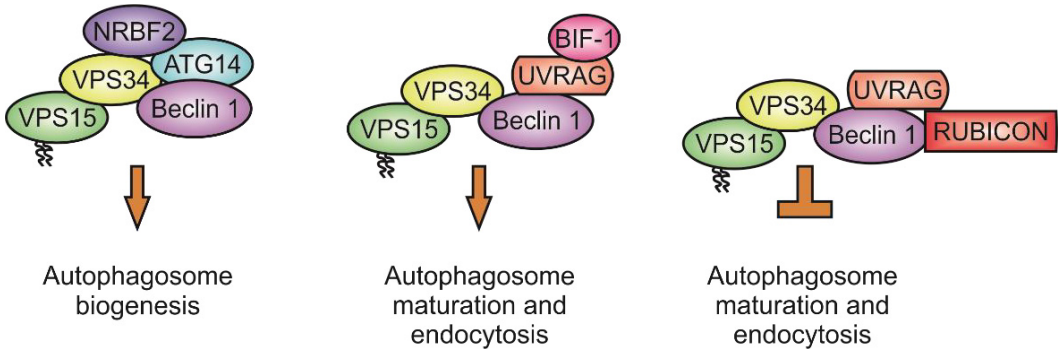

***Figure 6**: Schematic representation of mammalian Beclin 1 and the class III PI3K VPS34 complexes. Complex 1 (left) is involved in autophagosome biogenesis and contains Beclin 1, ATG14, VPS34 and VPS15. Complex 2 (middle) regulates autophagosome maturation and endocytic trafficking; in this complex, ATG14 is substituted by UVRAG. The interaction between UVRAG and Rubicon in complex 2 (right) inhibits autophagosome maturation and endocytosis.*

The core proteins of all these complexes are Beclin 1 itself, VPS34 and VPS15. Complex I includes, in addition to the aforementioned proteins, ATG14 and regulates autophagosome formation (Matsunaga et al. 2009, Zhong et al. 2009). In complex II, ATG14 is substituted by UVRAG; this complex regulates autophagosome maturation and plays a role in endocytosis (Thoresen et al. 2010). In the third complex, Rubicon (Run domain Beclin-1-interacting and cysteine-rich domain-containing protein) interacts with UVRAG and the other core components of complex II, inhibiting autophagosome maturation and endocytic trafficking (Thoresen et al. 2010). In the absence of Beclin 1, the core complex VPS34-VPS15 is highly unstable, causing impairment in VPS34 kinase activity, autophagy flux and endocytosis. Accordingly, in Beclin $1^{-/-}$ cells, VPS34 activity and autophagy flux were severely impaired (He et al. 2015).

Structural biology experiments show that the Beclin 1/VPS34 complex exhibits a V-shaped architecture (Baskaran et al. 2014). VPS15 assembles the complex by serving as a bridge for VPS34 and ATG14/Beclin 1 subcomplex. VPS15 is the pivot point of the complex and allows for dynamic transitions in which VPS34 is ejected from the complex in order to perform its catalytic activity. The N-terminus of Beclin 1 is located near the pivot point, playing a crucial role in the allosteric regulation of VPS34 lipid kinase activity.

1.2.3 Autophagy initiation: Beclin 1 interacting proteins in autophagosome biogenesis

As mentioned above, the PI3K complex I involved in autophagosome formation is assembled when Beclin 1 interacts with ATG14, VPS34 and VPS15. The fundamental mechanism by which Beclin 1 regulates autophagy relies on its allosteric control of VPS34 kinase activity (Kihara et al. 2001, Baskaran et al. 2014). VPS34 phosphorylates the three-position hydroxyl group of the inositol ring in phosphatidylinositol to generate PI3P (Backer 2016). PI3P is necessary for the recruitment of downstream autophagy effectors involved in autophagosome biogenesis. It has been shown that autophagy is severely impaired in the absence of VPS34 kinase activity (Petiot et al. 2000). VPS34 contains an N-terminal C2 domain, a middle helical domain and a C-terminal catalytic kinase domain (Figure 7) (Baskaran et al. 2014).

***Figure 7**: Schematic representation of VPS34 protein domain structures.*

VPS34 binds to Beclin 1 via the C2 domain. The lipid kinase domain at the C-terminus contains kα11 and kα12 helices necessary for its binding to VPS15. Moreover, the kα12 helix, and not kα11, must bind membranes in order to allow lipid phosphorylation of targets (Miller et al. 2010).

The PI3K complex I also contains the scaffold protein VPS15, essential for complex assembly as well as for VPS34 kinase activity (Stjepanovic et al. 2017). VPS15 protein contains an N-terminal serine/threonine kinase domain, a middle region with several HEAT repeats and a C-terminal WD40 domain (Figure 8).

***Figure 8**: Schematic representation of VPS15 protein domain structures.*

VPS15 is myristoylated at the N-terminus and this modification allows VPS15 to anchor to lipid membranes such as endosomal membranes and phagophores. This enables VPS34 translocation to these membranes and positively regulates VPS34 kinase activity.

ATG14 is also known as Beclin 1-associated autophagy-related key regulator (Barkor) (Itakura et al. 2008, Sun et al. 2008). ATG14 is thought to target the complex I to the site of autophagosome formation, sorting the Beclin 1 complex to specifically function in autophagy. ATG14 protein domains are shown in Figure 9.

***Figure 9**: Schematic representation of ATG14 protein domain structure. CCD (coiled-coild domain): BATS (Barkor/ATG14(L) autophagosome targeting sequence); ALPS (amphipathic lipid-packing sensor).*

The N-terminal CCD of ATG14 contains a cysteine-rich region necessary for its translocation, upon starvation, to the forming isolation membrane in association with the ER (Matsunaga et al. 2009). Moreover, the C-terminus of ATG14 is also involved in its targeting to the site of autophagosome formation via the hydrophobic surface of an ALPS (amphipathic lipid-packing sensor) motif contained within its BATS domain (Barkor/ATG14(L) autophagosome targeting sequence) (Fan et al. 2011).

1.2.4 Autophagy initiation: Regulation of Beclin 1 protein in autophagosome biogenesis

The function of Beclin 1 in autophagosome biogenesis is tightly regulated by various post-translational modifications such as phosphorylation, ubiquitination, proteolytic cleavage and acetylation (Hill et al. 2019). Phosphorylation is one of the most important and well-documented protein modifications in autophagy; this modification affects protein conformation, protein-protein interactions and enzymatic activity. The dissociation of Beclin 1 from Bcl-2 is important in order to allow Beclin 1-dependent autophagy induction. The interaction between Beclin 1 and Bcl-2 proteins represents the point of communication where the autophagic and apoptotic pathways meet (Pattingre et al. 2005). Wild-type Bcl-2 binds to Beclin 1 and inhibits Beclin 1-

dependent autophagy both in yeast and mammalian cells (Pattingre et al. 2005). Moreover, a Beclin 1-mutant unable to bind Bcl-2 protein induces excessive autophagy and promotes cell death. The death-associated protein kinase (DAPK) regulates Beclin 1-Bcl-2 interaction: DAPK phosphorylates Beclin 1 on Thr119 located within its BH3 domain, promoting the dissociation of Beclin 1 from Bcl-2 and inducing autophagy (Zalckvar et al. 2009). Beclin 1 Ser90 is an important phosphorylation site. Upon glucose withdrawal, AMPK phosphorylates Beclin 1 on Ser90 (and Ser94), regulating the VPS34 kinase activity and inducing autopha-gosome formation (Kim et al. 2013). Upon serum starvation, DAPK3 phosphorylates Beclin 1 on Ser90 in mouse skeletal muscle tissues, promoting autophagy (Fujiwara et al. 2016). Moreover, Ser90 is dephosphorylated by protein phosphatase 2A (PP2A).

Ubiquitination of proteins plays an important role in targeting them for degradation. In addition to this, ubiquitination can alter protein localization, protein-protein interactions and protein activities within multiprotein complexes. Ubiquitination of Beclin 1 at Lys117 modulates its interaction with Bcl-2, regulating autophagy induction (Shi and Kehrl 2010). The ubiquitin ligase NEDD4 polyubiquitinates the protein Beclin 1 with Lys11- and Lys63-linked chains, inducing proteasomal degradation of Beclin 1 and negatively regulating autophagy (Platta et al. 2012). Controversially, another study showed that NEDD4 positively regulates autophagy (Pei et al. 2017). In this study, NEDD4 promoted Lys6- and Lys27-linked ubiquitination of Beclin 1, protecting it from Lys48-linked polyubiquitination and targeting to proteasomes, and stabilizing its protein levels available for autophagy induction. The tripartite motif 50 (TRIM50) acts as an E3 ligase and polyubiquitinates Beclin 1 via Lys63-linked ubiquitin chains, enhancing its binding to ULK1 and positively regulating starvation- or rapamycin-induced autophagy (Fusco et al. 2018).

Proteolytic cleavage of Beclin 1 adds another layer of regulation to Beclin 1 activity in autophagy. Beclin 1 contains two cleavage sites for caspase 3, $TDVD^{133}$ and $DQLD^{149}$ (Wirawan et al. 2010). The generated fragments lack autophagic activity. Moreover, the C-terminal Beclin 1 fragment relocates to the mitochondria, enhancing apoptotic response by the cell.

Lastly, acetylation also plays a role in Beclin 1 regulation. Beclin 1 is acetylated by histone acetyltransferase EP300 at Lys430 and Lys437. This promotes Beclin 1 binding to Rubicon, inhibiting both autophagosome maturation and endocytic trafficking (Sun et al. 2015).

The coordinated regulation of the protein Beclin 1 is in line with its crucial role in autophagy initiation. More importantly, it could be used as a potential therapeutic target, for example by targeting Beclin 1 post-translational modification sites to manipulate the autophagy response in different disease settings.

In addition to its role in autophagy, Beclin 1 mediates multiple other non-autophagic cellular processes (Galluzzi and Green 2019). As mentioned before, Beclin 1 positively regulates endocytosis (see chapter 1.2.2 for details). Beclin 1 is also involved in a specific form of phagocytosis, known as LC3-associated phagocytosis (LAP), which is induced in response to different pathogens, including invasion by the fungal pathogen *Aspergillus fumigatus* (Martinez et al. 2015). It has been shown that Beclin 1 plays a role during oocyte meiosis independently of ATG14 (You et al. 2016). Beclin 1 formed small vesicles during oocyte meiosis and was also seen on midbodies during cytokinesis. Cytokinesis refers to the step in cell division where the parent cell is separated into daughter cells.

1.2.5 Phosphatidylinositol-3-phosphate-binding proteins and ATG2

The PI3P produced by the Beclin 1/VPS34 complex binds to several downstream autophagy effectors. The first effector that binds to PI3P during autophagosome biogenesis is the omegasome-resident protein DFCP1 (double-FYVE-containing protein 1) (Axe et al. 2008). DFCP1 does not have an essential role in autophagy and its absence does not affect autophagy flux.

WIPIs (WD-repeat domain phosphoinositide-interacting proteins), members of the PROPPIN family, are also PI3P effectors. There are four mammalian WIPIs and WIPI1 and WIPI2 were the first members of the family to be shown to gather at the site of autophagosome formation, specifically on phagophore membranes (Polson et al. 2010). WIPI2 positively regulates autophagy and is required for the formation of LC3-positive autophagosomes in omegasomes. Moreover, WIPI2 directly interacts with ATG16, positively regulating LC3 lipidation and autophagy flux (Dooley et al. 2014).

ATG2A and ATG2B are the mammalian homologues for the yeast Atg2 (Velikkakath et al. 2012). They are required for autophagy and display overlapping functions. ATG2A localizes on isolation membranes and lipid droplets and depletion of both ATG2A and ATG2B blocks autophagy flux and causes the accumulation of open autophagic structures (Velikkakath et al. 2012). Moreover, silencing of ATG2A/B increases the number and size of lipid droplets and induces their clustering in the cell

(Velikkakath et al. 2012). It has been shown that WIPI4 interacts with ATG2A and ATG2B (Chowdhury et al. 2018). ATG2A localizes on PI3P-enriched phagophores and forms a complex with WIPI4 that mediates ER-phagophore association and tethers membrane vesicles for phagophore elongation (Chowdhury et al. 2018). Recent results show that ATG2 is a novel phospholipid transfer protein that likely mediates lipid transport to forming phagophores (Osawa et al. 2019, Osawa and Noda 2019, Valverde et al. 2019).

1.2.6 Phagophore elongation: ATG12-ATG5 and ATG8-PE conjugation systems

Phagophore expansion relies on the essential activity of two ubiquitin-like conjugation systems: the ATG12-ATG5 system and the ATG8-phosphatidylethanolamine (ATG8-PE) system (Figure 10). Although yeast has only one Atg8, mammals have several ATG8 homologs (Lee and Lee 2016). These homologs include the following: LC3, also known as MAP1LC3 (microtubule associated protein 1 light-chain 3), which has A, B and C variants; GABARAP (γ-aminobutyric acid receptor-associated protein), GABARAPL1 (γ-aminobutyric acid receptor-associated protein like 1) and GABARAPL2.

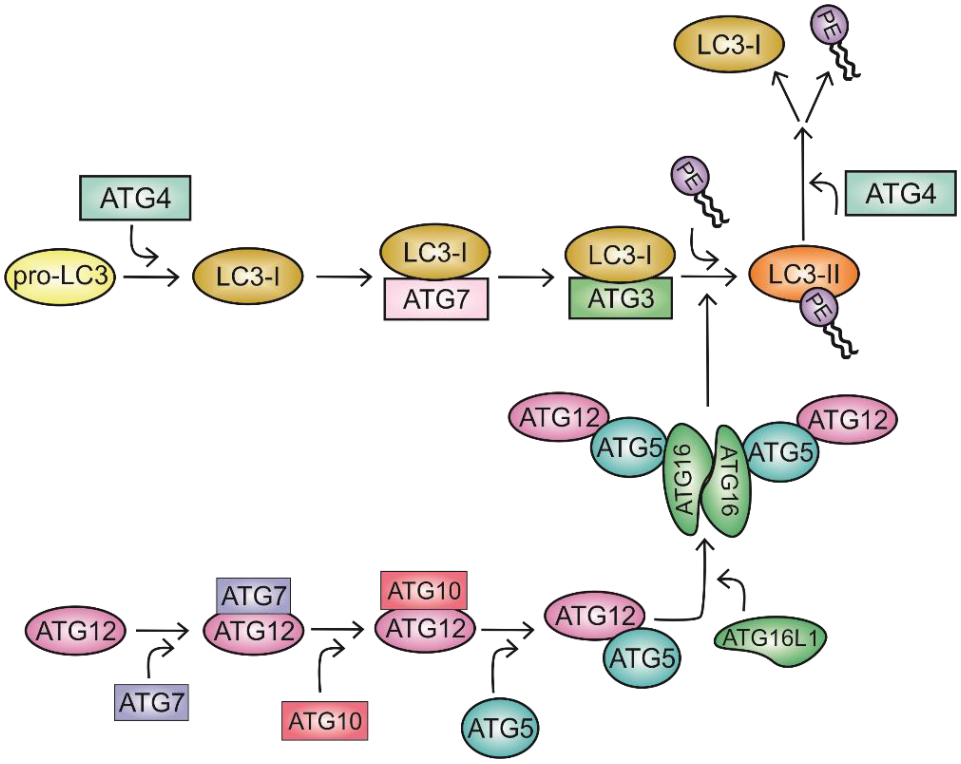

***Figure 10**: Schematic representation of the ATG12-ATG5 and LC3-PE conjugation systems (see text for details). GABARAPs are lapidated similar to LC3s.*

ATG12 is activated by ATG7 (E1-like enzyme), and subsequently transferred to ATG10 (E2-like enzyme) to form a covalent conjugate with ATG5 (Mizushima et al. 1998). The ATG12-ATG5 conjugate then forms a complex with ATG16 with a stoichiometry of 2:2:2 via homodimerization of the protein ATG16. The ATG12-ATG5-ATG16 complex is assembled constitutively but localizes to the outer surface of the forming phagophore and leaves the site right before or soon after the completion of the autophagosome (Mizushima et al. 2001). It has been suggested that the interaction between ATG16L1 and the heavy chain of clathrin plays a role in the formation of early autophagosome precursors positive for ATG16 (Ravikumar et al. 2010). Inhibition of clathrin-coated vesicle generation also impaired ATG16 positive structures as well as the appearance of autophagosomes. Notably, this study set the plasma membrane as a possible membrane source for phagophore precursors.

The ATG8, exemplified by LC3-PE, conjugation system is recruited downstream of the ATG12-ATG5-ATG16 complex (Geng and Klionsky 2008). The unique feature here is the conjugation of LC3 to a lipid and not a protein. LC3-PE was originally thought to be needed for phagophore membrane elongation and closure of the forming autophagosome membrane (Kabeya et al. 2000). LC3 resides on both the inner and outer autophagosomal membranes via its conjugation to the lipid PE. Pro-LC3 is processed by the protease ATG4 to its cytosolic form called LC3-I (Figure 10). This is followed by sequential steps including interaction of LC3-I with ATG7 (E1-like enzyme) first and ATG3 (E2-like enzyme) next; lastly, the ATG12-ATG5-ATG16 complex (E3-like enzyme) generates the membrane-bound LC3-II via interaction with ATG3, leading to conjugation of LC3 to PE (Kabeya et al. 2000, Hanada et al. 2007, Fujita et al. 2008). When the autophagosome fuses with the lysosome, the inner membrane-bound LC3-II is degraded together with the cargo via the lysosomal hydrolytic enzymes and the outer membrane-bound LC3-II is released back to the cytoplasm by delipidation to its cytoplasmic form by ATG4. There is a close crosstalk between the two ubiquitin-like systems. The ATG5-ATG12-ATG16 conjugate acts as an E3-like enzyme for LC3 lipidation on autophagosomal membranes. ATG3 recognizes ATG12, which allows the ATG5-ATG12-ATG16 complex to function as an E3-like enzyme mediating and accelerating the formation of the LC3-PE conjugate (Hanada et al. 2007, Fujita et al. 2008).

1.2.7 Phagophore elongation: ATG4 and its role in the LC3-PE conjugation system

One of the key players in the LC3-PE conjugation system is the cysteine protease ATG4 (homologous to yeast Atg4). There are four mammalian ATG4 proteins: ATG4A, ATG4B, ATG4C and ATG4D (Li et al. 2011). ATG4B has the highest proteolytic activity towards LC3 and GABARAP subfamilies (GABA type A receptor-associated protein) while ATG4A is active only towards GABARAP. ATG4C and ATG4D are mostly inactive. Knockout of ATG4B in HeLa cells inhibits LC3 lipidation and autophagic flux (Fu et al. 2018), and studies with expression of an inactive mutant form of ATG4B showed a clear defect in autophagosome closure and strong inhibition of autophagy flux due to sequestration of ATG4B-bound LC3 in the cytosol (Fujita et al. 2008). The first step in LC3-PE conjugation is the proteolytic cleavage of pro-LC3 via ATG4B that exposes a glycine residue near the C-terminus and generates the LC3-I form. Following autophagosome closure, ATG4B delipidates LC3-II from the outer autophagosomal membrane by cutting the bond between the C-terminal carboxyl moiety and the amine group of PE. The regulation of the ATG4-mediated delipidation step is still quite elusive. Deconjugation of LC3-PE via ATG4 is important

for recycling a pool of LC3 for the next conjugation round and the formation of new autophagosomes (Nakatogawa et al. 2012). The regulation of this step must allow for delayed delipidation until the autophagosome is completed: if the deconjugating activity of ATG4 would be too high, there would be no LC3-PE to function in autophagosome biogenesis.

Recent studies have shown that ATG4 activity is regulated by a series of post-translational modifications. ATG4A and ATG4B activity is regulated by reactive oxygen species (ROS) by oxidation of Cys81 and Cys78 (Scherz-Shouval et al. 2007). This redox regulation of ATG4 is important for fast activation-inactivation cycles of the protease. Phosphorylation of ATG4B in different sites has been shown to play an important role in its regulation. ATG4B is a substrate of the serine/threonine kinase MST4 (mammalian STE20-like protein kinase 4). Upon radiation, ATG4B is phosphorylated on Ser383 which stimulates ATG4B activity and enhances the autophagy response in glioblastoma cells (Huang et al. 2017). Interestingly, also ULK1 kinase has been shown to regulate ATG4B activity (Pengo et al. 2017). ULK1 phosphorylates ATG4B on Ser316, inhibiting its catalytic activity both in vivo and in vitro. Moreover, the phosphatase PP2A-PP2R3B can dephosphorylate this site, eliminating the ULK1-mediated inhibition. The mechanisms underlying this paired regulation via ULK1-mediated phosphorylation and PP2A-mediated dephosphorylation are not fully understood, but it provides an effective phospho-switch regulating the cellular activity of ATG4B in LC3 processing. Ubiquitination also plays a role in ATG4B regulation. The membrane-associated E3 ligase RNF5 (ring finger protein 5) regulates ATG4B activity by targeting it for ubiquitination and proteasomal degradation thus downregulating autophagy via reduced LC3 processing (Kuang et al. 2012).

It has been reported that ATG4D plays a role in apoptosis in addition to its function in autophagy. During apoptosis, ATG4D is cleaved by caspase-3 at the N-terminal DEVD^{63}K cleavage site (Betin and Lane 2009, Betin and Lane 2009). Full length ATG4D was shown to be enzymatically inactive; upon caspase-3-mediated cleavage, truncated ATG4D (ΔN63 Atg4D) became active towards GABARAPL1, allowing its lipidation and delipidation. Silencing of ATG4D reduced GABARAPL1 puncta (representing autophagosomes) both in nutrient-rich conditions and under starvation (Betin and Lane 2009, Betin and Lane 2009). Next to the caspase cleavage site, ATG4D contains a mitochondrial targeting sequence (Betin et al. 2012). Upon caspase cleavage, ATG4D is imported into mitochondria where it is further processed. Mitochondrial ATG4D induces cell death in the presence of the mitochondrial uncoupler CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) (Betin et al. 2012).

Moreover, cells expressing the mitochondrially cleaved ATG4D form show reduced mitochondrial cristae density and altered mitochondrial function.

Impaired activity of ATG4D has also been implicated in various diseases. Defective expression of ATG4D has been suggested to play a role in the pathogenesis of human uterine fibroids (UF) (Andaloussi et al. 2017). UF tissues displayed a blockage in autophagosome maturation and autophagy flux. Moreover, a recessive missense mutation in the ATG4D gene (c.1288G>A; p.A430T) is implicated in a neurodegenerative disorder found in Lagotto Romagnolo (LR) dogs (Kyöstilä et al. 2015). The affected dogs exhibited progressive cerebellar ataxia, aberrant neuronal cytoplasmic vacuolization and altered autophagy flux in the nervous system and in secretory epithelial tissues.

1.3 The origin of the autophagosome membrane

One of the key questions in the field of autophagy, that still lacks a clear answer, is the origin of the phagophore membrane. One of the major problems in addressing this question has been the lack of specific membrane-spanning protein markers within autophagic structures. Instead, most of the known autophagy proteins available as organelle markers only transiently associate with membranes during autophagosome biogenesis. Moreover, since the autophagosome content reflects the cellular cytoplasm, isolating possible membrane sources, phagophores or autophagosomes, using subcellular fractionation, is also problematic (Tooze and Yoshimori 2010). The special nature of autophagosome membranes was first discovered when researchers applied cytochemical techniques in order to identify the origin and structure of autophagic membranes (Reunanen et al. 1985). In this study, the phagophore was stained with reduced osmium tetroxide, revealing a different staining pattern compared to other subcellular organelles.

Numerous studies have shown that the endoplasmic reticulum (ER) is strongly linked to autophagosome biogenesis (Ktistakis 2019). ER was the first organelle proposed as a source for autophagosome biogenesis through electron microscopy studies (Novikoff and Shin 1978). This study showed that autophagic vacuoles are continuous with ER. Immuno-ultrastructural observations showed the presence of integral membrane proteins of the rough ER within autophagic structures (Dunn 1990). Despite these results, a question still remained in the field: is the membrane formed *de novo* or from a pre-existing organelle(s) (Eskelinen et al. 2011)? The Golgi complex, plasma membrane, endosomes and lysosomes were thought to represent

the pre-existing organelles that could give rise to autophagosomes. However, opposing results from different studies showed either the presence or absence of Golgi or ER markers in autophagic structures, as well as physical differences between autophagic membranes and the membranes of surrounding organelles. The discovery of the autophagy-related (ATG) genes in yeast provided the missing protein markers to study the molecular machinery driving the autophagy process. Currently, LC3 (microtubule-associated proteins 1A/1B light chain 3) is the major autophagosomal marker used to follow autophagosome formation and maturation. It was used to show for the first time that autophagosomes are indeed distinct from other subcellular compartments (Kabeya et al. 2000). Live-cell studies confirmed the prominent role of the ER in autophagosome biogenesis by showing that phagophores emerge from a phosphatidylinositol 3-phosphate (PI3P) enriched ER subdomain, called the omegasome (Axe et al. 2008): omegasomes are positive for the PI3P-binding and ER-resident protein DFCP1. Electron tomography studies shed more light on the close relationship of the ER with autophagosome biogenesis. Two independent studies showed that the forming autophagosome is sandwiched between ER sheets and revealed the existence of physical connections between the ER and the forming autophagosome (Hayashi-Nishino et al. 2009, Yla-Anttila et al. 2009). These studies supported the omegasome hypothesis, where the omegasome and the underlying ER act as a cradle for autophagosome biogenesis. Another study used cryo-fluorescence and cryo-soft X-ray microscopy to show that omegasomes emerge from the ER (Duke et al. 2014). Groups of omegasomes seemed to form on small ER “hot regions” suggesting possible connections within them during autophagosome biogenesis.

Despite the huge amount of data supporting the ER as a major membrane source for autophagosome biogenesis, several other organelles have been implicated in the past years as possible membrane sources (Molino et al. 2017). Among them, mitochondria, ER-mitochondria contact sites, the plasma membrane, recycling endosomes and the Golgi complex all seem to be involved in autophagosome biogenesis either via direct interaction with the forming autophagosome or by accommodating proteins involved in the molecular regulation of autophagy (Hailey et al. 2010, Ravikumar et al. 2010, Orsi et al. 2012, Hamasaki et al. 2013, Biazik et al. 2015).

1.4 Signalling in starvation induced autophagy

Nutrients such as glucose and amino acids as well as oxygen and adenosine triphosphate (ATP) are vital for cellular metabolism (Galluzzi et al. 2014, Russell et al. 2014). Cells need to respond efficiently to fluctuations in their levels in order to

maintain a balance between anabolic and catabolic reactions. There are two degradative pathways in the cell: autophagy and the ubiquitin-proteasome system. The major difference between them relies on the fact that autophagy produces energy in the form of nutrients, in contrast to the ubiquitin-proteasome system that requires ATP for degradation. Further, autophagy is the sole pathway able to degrade whole organelles or large protein aggregates. That is why autophagy is upregulated in response to nutrient depletion. It has been shown that ATG5- or ATG7-null mice die 24 hours after birth due to a severe defect in autophagy leading to amino acid deficiency and decreased glucose levels (Kuma et al. 2004, Komatsu et al. 2005).

As discussed above, mTORC1 is the kinase that senses cellular nutrient levels. One of the important activators of mTORC1 is intracellular amino acid level (Bar-Peled and Sabatini 2014). In nutrient-rich conditions, mTORC1 resides on the lysosomal surface (Sancak et al. 2008). One of the downstream targets of mTORC1 is the ULK1 complex whose activity is regulated by phosphorylation events that target ULK1 itself and its binding partners (see chapter 1.2.1 for details). Interestingly, mTORC1 interacts also with the ATG14-containing Beclin 1 complex (Yuan et al. 2013). In nutrient-rich conditions, mTORC1 phosphorylates ATG14 on five different phosphorylation sites, inhibiting the activity of the Beclin 1 complex and autophagy induction. The mTORC1-mediated regulation of the ULK1 and Beclin 1 complexes demonstrates the importance of amino acid fine-tuning for mammalian autophagy initiation.

Cellular energy levels are regulated by the AMPK, a serine/threonine kinase that senses cellular ATP:ADP:AMP ratios (Hardie 2011). Upon ATP withdrawal, AMPK enhances energy production through glucose uptake and glycolysis. As mentioned above, one of the downstream effectors AMPK is the ULK1 kinase (see chapter 1.2.1 for details). Moreover, AMPK also phosphorylates Beclin 1 and VPS34 in the Beclin 1 complexes containing ATG14 or UVRAG (Kim et al. 2013). AMPK inhibits the activity of the UVRAG-containing Beclin 1 complex via phosphorylation of Thr163 and Ser165 on VPS34. Upon glucose withdrawal, AMPK phosphorylates Beclin 1 on Ser90 and Ser94, inducing autophagosome formation (Kim et al. 2013). Another study showed that, upon glucose starvation, AMPK also phosphorylates Beclin 1 on Thr388, disrupting Beclin 1-Bcl-2 interaction and inducing autophagy (Zhang et al. 2016). Interestingly, ULK1 phosphorylates AMPK and negatively regulates its activation and pro-autophagic activity (Loffler et al. 2011). This demonstrates the existence of a negative regulatory loop between ULK1 and AMPK that contributes to the termination of the signaling events leading to the autophagic response.

The inactive form of the oncogenic receptor tyrosine kinase EGFR (epidermal growth factor receptor) has been suggested to play a role in starvation-induced autophagy (Tan et al. 2015). Inactive EGFR forms a complex with the oncoprotein LAPTM4B

(lysosomal protein transmembrane 4B) and with Sec 5 (also known as exocyst complex component 2, EXOC2) localizing on endosomes. This complex promotes EGFR binding to Rubicon, dissociating the latter from Beclin 1 in order to initiate autophagy response.

Amino acid starvation-induced autophagy is also positively regulated by the stress-related kinases MK2 and MK3, members of the p38 mitogen-activated protein kinase (MAPK) signaling pathway (Wei et al. 2015). Upon starvation, MK2 and MK3 directly phosphorylate Beclin 1 on Ser90. A mutation in this phosphorylation site inhibits the autophagic response and suppresses the oncosupressor activity of Beclin 1 in MCF7 breast cancer cells.

1.5 Selective autophagy

It is widely accepted that autophagy is active at a low basal level that is independent of nutrients and stress status. Basal autophagy is important for cellular homeostasis and is also referred to as quality control (QC) autophagy (Murrow and Debnath 2013).

Comprehensive studies have shed light on the highly selective nature of basal autophagy (Mancias and Kimmelman 2016, Zaffagnini and Martens 2016). Selective autophagy targets and degrades whole organelles, invading pathogens and large protein aggregates.

Effective selective autophagy needs to meet three criteria. (Mancias and Kimmelman 2016, Zaffagnini and Martens 2016). First, the degradative cargo must be specifically recognized by “selective autophagy receptors”. This should be followed by the tethering of the cargo to a forming autophagosome. Lastly, there should be a regulatory mechanism that excludes non-cargo material from degradation.

Selective autophagy receptors are necessary for correct cargo recognition as they link together the forming autophagosome and the cargo through interaction with LC3-GABARAP-like proteins on the autophagosomal membranes (Stolz et al. 2014). The selective receptors interact with LC3-GABARAP-like proteins through LC3-interacting regions (LIR) and are tightly regulated to guarantee efficient and fast cargo degradation (Rogov et al. 2014). Selective autophagy shares the molecular machinery regulating canonical autophagy that was described earlier: ULK1, for example, directly binds to LC3 via the LIR region, ensuring the correct localization of the autophagy machinery and cargo degradation (Rui et al. 2015).

Several types of selective autophagy have been discovered and they are all named after the cargo intended for degradation: mitophagy (mitochondria), aggrephagy

(protein aggregates), reticulophagy/ER-phagy (ER), lipophagy (lipid droplets), lysophagy (lysosomes), ferritinophagy (ferritin), nucleophagy (nuclear envelope) and xenophagy (pathogens). They are divided in two major groups: ubiquitin-dependent and ubiquitin-independent cargo recognition (Table 2, Table 3 and Figure 11) (Khaminets et al. 2016).

***Table 2**: Ubiquitin-dependent selective autophagy receptors, modified from (Khaminets et al. 2016)*

Pathway	Receptors	Cargo	Reference
Mitophagy	OPTN, p62, NDP52, TAX1BP1	Mitochondria	(Wong and Holzbaur 2014, Heo et al. 2015, Lazarou et al. 2015)
Pexophagy	p62, NBR1	Peroxisomes	(Deosaran et al. 2013)
Aggrephagy	p62, NBR1, OPTN, TOLLIP, Cue5	Protein aggregates	(Pankiv et al. 2007, Kirkin et al. 2009, Korac et al. 2013, Zhou et al. 2013, Lu et al. 2014)
Xenophagy	p62, OPTIN, NDP52	Bacteria	(Thurston et al. 2009, Zheng et al. 2009, Wild et al. 2011)

***Table 2**: Ubiquitin-dependent selective autophagy receptors, modified from (Khaminets et al. 2016) (continued)*

Pathway	Receptors	Cargo	Reference
Nucleic acid disposal	p62, NDP52	Nucleic acids	(Watson et al. 2012, Guo et al. 2014)
Zymophagy	p62	Zymogen granules	(Grasso et al. 2011)
Proteophagy	RPN10	Proteasomes	(Marshall et al. 2015)
Midbody degradation	p62, NBR1	Midbody	(Pohl and Jentsch 2009, Kuo et al. 2011)

***Table 3**: Ubiquitin-independent selective autophagy receptors, modified from (Khaminets et al. 2016)*

Pathway	Receptors	Cargo	Reference
ER-phagy	FAM134	ER	(Khaminets et al. 2015, Mochida et al. 2015)
Ferritinophagy	NCOA4	Ferritin	(Dowdle et al. 2014)
Mitophagy	NIX, BNIP3, FUNDC1, Atg32	Mitochondria	(Kanki et al. 2009, Zhang and Ney 2009, Chen et al. 2016)
Glycophagy	Stbd1	Glycogen	(Jiang et al. 2011)
Xenophagy	Galectin-8	Bacteria	(Thurston et al. 2012)
Nuclear lamina autophagy	Lamin B1	Nuclear lamina/nuclear envelope	(Dou et al. 2015)

***Table 3**: Ubiquitin-independent selective autophagy receptors, modified from (Khaminets et al. 2016) (continued)*

Pathway	Receptors	Cargo	Reference
Nucleophagy	Atg39	Nuclear envelope	(Mochida et al. 2015)
Pexophagy	NBR1, Atg30, Atg36	Peroxisomes	(Deosaran et al. 2013, Oku and Sakai 2016)
Signalophagy	c-Cbl	Src	(Sandilands et al. 2012)
Cvt targeting	Atg19, Atg34	Ape1, Ams1	(Watanabe et al. 2010)
Virophagy	TRIM5α, SMURF1	Viruses	(Orvedahl et al. 2011, Mandell et al. 2014)
Lysophagy	Galectin-8	Lysosomes	(Thurston et al. 2012)

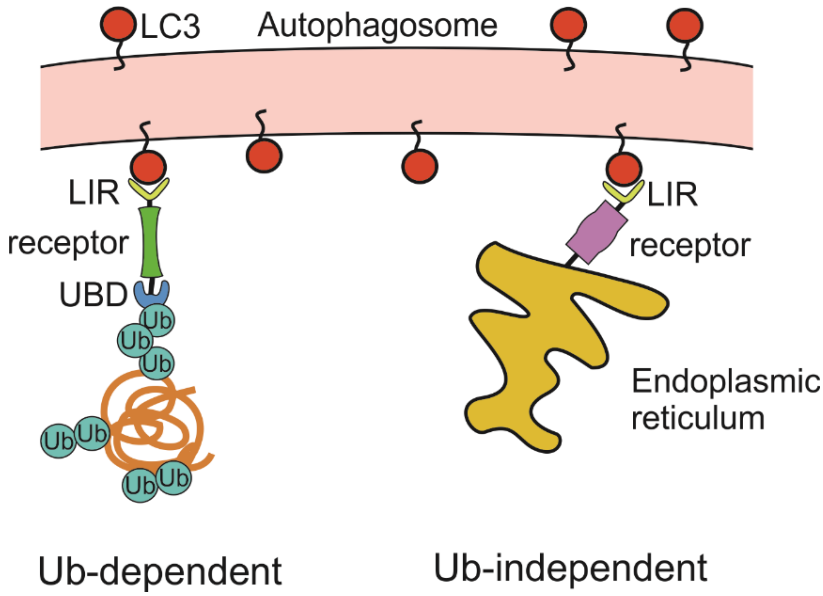

***Figure 11**: Schematic representation of ubiquitin (Ub)-dependent and ubiquitin-independent selective autophagy. In the Ub-dependent selective autophagy (left), the autophagy receptor (green) recognizes the ubiquitin-bound (light blue) cargo through the Ub-binding domains (UBD, blue) and interacts with LC3 via the LC3-interacting region (LIR, yellow). In the ubiquitin-independent selective autophagy (right), the autophagy receptor (violet) directly binds to the cargo.*

1.5.1 Ubiquitin-dependent selective autophagy and p62

Ubiquitination of proteins is involved in different cellular processes. Protein molecules that are modified with polyubiquitin chains are targeted for proteosomal degradation. Ubiquitin also plays a pivotal role in selective autophagy since it acts as a molecular tag for cellular cargo to be selectively degraded by autophagy. Aberrant protein aggregates, or other stress effectors, are ubiquitylated and recognized by selective receptors such as p62, also called SQSTM1 (sequestosome 1), via their ubiquitin-binding domains (UBD) (Pankiv et al. 2007, Lamark et al. 2009). This tethers the cargo to the forming autophagosome by interacting with LC3 or other ATG8 homologs on the autophagosomal membranes. The autophagosome then delivers the cargo to the lysosome for degradation. The p62 protein itself is a substrate for selective autophagy. It has several domains: the PB1 oligomerization domain, involved in the oligomerization of ubiquitylated aggregates; the ZZ-type zinc finger domain; the LIR domain for LC3-p62 interaction; the KEAP1-interacting region (KIR) and the ubiquitin-

associated domain (UBD) which binds mono-ubiquitin and polyubiqutin chains (Lamark et al. 2009, Mancias and Kimmelman 2016, Johansen 2019).

One of the best characterized selective autophagy pathways is the selective degradation of large protein aggregates (Hyttinen et al. 2014). A protein aggregate is formed when misfolded proteins aggregate together and form insoluble clumps, called aggresomes. Aggresomes can be deleterious to the cell and can eventually cause cellular death. Several studies have linked the appearance of aggregates to various neurodegenerative diseases, including Alzheimer´s disease (AD), Parkinson´s disease (PD), Huntington´s disease (HD) and prion diseases. NBR1 and p62 are both required for the selective degradation of misfolded proteins via aggrephagy (Lamark et al. 2009). p62 is also involved in selective degradation of ubiquitylated bacteria, also known as xenophagy (Thurston et al. 2009). OPTN (optineurin) and NDP52 (nuclear dot protein 52 kDa) are the main selective autophagy receptors that target invading bacteria to the autophagy pathway (Thurston et al. 2009, Zheng et al. 2009). Ubiquitination of mitochondrial outer membrane proteins activates selective degradation of mitochondria via autophagy (mitophagy). Optineurin, p62, NDP52 and TAX1BP1 (Tax1-binding protein 1) are all involved in the recognition of polyubiquitylated mitochondrial outer membrane proteins prior to mitochondrial degradation via autophagy (Wong and Holzbaur 2014, Heo et al. 2015, Lazarou et al. 2015).

1.5.2 TRIMs and precision autophagy

The tripartite motif (TRIM) family proteins are involved in several cellular processes such as immunity, cell proliferation, DNA repair, transcription, cell cycle progression, inflammation and apoptosis (Hatakeyama 2017, Gushchina et al. 2018, Vunjak and Versteeg 2019). TRIMs are characterized by common structural protein domains that are important for their functions (Figure 12) (Esposito et al. 2017).

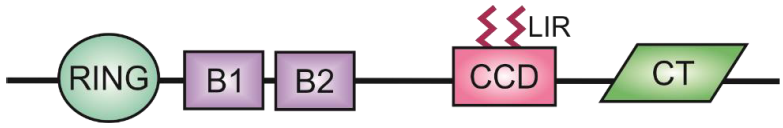

***Figure 12**: Schematic representation of the TRIM protein domains. TRIM proteins contain a RING domain, followed by one or two B-box domains and a Coiled Coil Domain (CCD). The CCD contains LC3-interacting regions (LIR). The C-terminal (CT) domain is usually used to categorize the TRIMs family member into subgroups.*

TRIM proteins contain a Really Interesting New Gene (RING) domain at the N-terminus, followed by one or two zinc-finger domains called B-boxes (B1 box and B2 box) with their related coiled-coil domain (CCD). The CCD contains LC3-interacting regions (LIRs). TRIM proteins have been classified as E3 ubiquitin ligases due to the presence of the RING finger that acts as the catalytic centre of the protein. However, it has been shown that eight TRIM proteins have no RING domain (Hatakeyama 2017). The C-terminal domain of TRIM proteins is very variable and contains different protein-protein interaction domains such as the COS domain, acid-rich region (ACID), PRY domain, SPRY domain, filamin-type IG domain (FIL), NHL domain, PHD domain, bromodomain (BROMO), Meprin and TRAF-homology domain (MATH) and transmembrane region (TM) (Hatakeyama 2017). The variable nature of the C-terminus allows the classification of the TRIM family proteins into 11 sub-families, named from C-I to C-XI with the addition of a UC sub-family lacking the RING domain. TRIM proteins can form homo-oligomers with themselves as well as hetero-oligomers with other members of the TRIM family via the B-box and CCD domains. TRIM proteins form an anti-parallel homodimer through their B-box and CCD domains, separating the catalytic RING domains at opposite sides of the dimer for concurrent cargo ubiquitination (Koliopoulos et al. 2016).

As mentioned before, TRIM proteins are involved in various cellular processes, and an increasing number of studies have shown that they can bind to several different substrates via the variable C-terminal domains. Innate immunity is one of the best characterized cellular processes where TRIM proteins play a major role. One of the first studies on the relationship between TRIMs and innate immunity showed that TRIM5α plays a role in the recognition and proteasome targeting of several viruses, including retroviruses and lentiviruses, preventing infection (Stevenson 2004). TRIM21 is a cytosolic IgG receptor that binds with high affinity to IgG, IgA and IgM found on invading pathogens, adds ubiquitin on them via E3 ubiquitin ligase activity, and thus targets them for degradation via autophagy (Mallery et al. 2010). TRIM19, a component of nuclear bodies, plays a role in viral infection (Rajsbaum and Garcia-Sastre 2013). Several viruses encode proteins that interact with TRIM19, causing disruption of the nuclear bodies and impaired antiviral response.

TRIM proteins play a role also in carcinogenesis. TRIM17 interacts and promotes the degradation of the kinetochore protein ZWINT that is involved in the proliferation of breast cancer cells (Horie-Inoue 2013). It has also been shown that TRIM17 expression is abnormal in certain types of cancers compared to other TRIM family members. This imbalance causes disturbed ubiquitination of target substrates, and triggers altered cellular signalling and tumorigenesis. Many other TRIM proteins (TRIM8, TRIM13, TRIM19, TRIM24, TRIM25, TRIM31, TRIM33, etc.) are involved in

cancer progression via changes in protein expression levels or chromosomal translocation (Hatakeyama 2011).

TRIM proteins have also been shown to play an important role in autophagy. TRIM55, a muscle-specific ubiquitin ligase also known as MuRF2, was the first member of the TRIM family to be shown to regulate autophagy by interacting with the autophagy receptor p62 (Lange et al. 2005). TRIM55 regulates the assembly of sarcomers and protein degradation in striated muscles. Another study showed that, upon ER stress, TRIM13 induced autophagy by interacting with p62 via its coiled-coil domain (Tomar et al. 2012). TRIM13 colocalized with the omegasome protein DFCP1 and promoted autophagy initiation. TRIM28 was shown to recruit and interact with acetylated hsp70. The resulting complex mediated SUMOylation of VPS34, which increased VPS34 activity and its binding to Beclin 1, inducing autophagosome formation (Yang et al. 2013).

Interestingly, TRIM proteins seem to have a dual role in autophagy. They act as autophagy receptors by binding to cellular degradative cargo and, in addition, as autophagy regulators by binding to core autophagy proteins thus regulating autophagy initiation (Mandell et al. 2014, Kimura et al. 2015, Kimura et al. 2016). TRIM proteins first recognize their cargo and directly interact with it without the need of ubiquitin (Mandell et al. 2014, Kimura et al. 2015). Next, core autophagy regulators such as ULK1, Beclin 1 and ATG16 assemble on TRIM proteins and induce autophagosome formation for cargo degradation. This TRIM-containing complex, formed in connection with the growing phagophore membrane, has been called TRIMosome (Figure 13) (Mandell et al. 2016, Hatakeyama 2017). The mechanism by which TRIMs both regulate autophagosome formation and act as selective autophagy receptors has been named “precision autophagy” (Kimura et al. 2016).

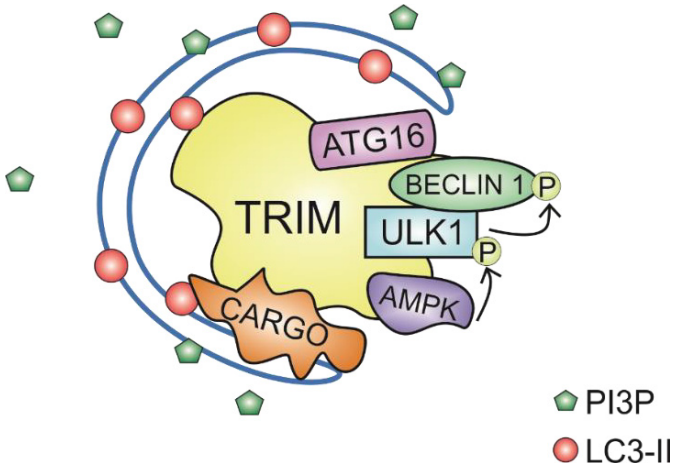

***Figure 13**: Schematic representation of the TRIMosome (see text for details).*

In the TRIMosome, TRIM proteins interact with cellular stressors via the variable C-terminus, allowing a broad spectrum of cargo recognition. They can target both endogenous and exogenous cargo for degradation through autophagy. TRIM20 recognizes and targets the inflammasome components NLRP1 and NLRP3 (NLR-family pyrin-domain-containing proteins 1 and 3) and pro-caspase 1 for autophagy degradation (Kimura et al. 2015). TRIM21 targets interferon regulatory factor 3 (IRF-3), a transcription factor regulating gene expression of type-I interferon (IFN), for autophagy degradation (Kimura et al. 2015). Several TRIMs bind to LC3 or GABARAP proteins through their LIRs, linking the TRIMosome to the forming phagophore. Studies in vitro have shown that TRIM16, TRIM17, TRIM22, TRIM49 and TRIM55 interact with LC3 (Mandell et al. 2014).

TRIM proteins can be either positive or negative regulators of autophagy. For example, TRIM17 negatively regulates autophagy induced by the mTORC1 inhibitor pp242 by sequestering the autophagy machinery (Mandell et al. 2014). TRIM17 also inhibits selective degradation of p62 while inducing selective autophagy of midbodies (Mandell et al. 2016). TRIM28 positively regulates autophagy by enhancing the binding of VPS34 to Beclin 1, while it downregulates autophagy by targeting AMPK for proteasomal degradation (Pineda and Potts 2015).

Several TRIMs, including TRIM5, TRIM6, TRIM17, TRIM21, TRIM22 and TRIM49, interact with core autophagy regulators such as Beclin 1 and ULK1 (Mandell et al.

2014, Kimura et al. 2015). TRIM5, TRIM13 and TRIM17 colocalize with DFCP1 (Tomar et al. 2012, Mandell et al. 2014). Several other autophagy effectors, such as FIP200, VPS34, ATG14, UVRAG, WIPI2, ATG16, ATG5 and ATG12 as well as AMBRA 1, have been detected in complexes with different TRIM proteins (Behrends et al. 2010, Yang et al. 2013, Mandell et al. 2014, Kimura et al. 2015).

1.6 Maturation of autophagosomes into degradative autolysosomes and autophagic lysosome reformation

Once formed, autophagosomes fuse with vesicles originating from the endocytic pathway in order to eventually become degradative autolysosomes in a process known as autophagosome maturation. Autophagic degradation is known to occur only through lysosomal activity. Therefore, fusion of the autophagosome with the lysosome is a major step to correctly terminate the autophagic process.

1.6.1 Autophagosome maturation

Autophagosome maturation is defined as the stepwise process by which the nascent autophagosome grows into a degradative autolysosome. This must not be confused with the maturation of phagophores membranes into closed autophagosomes. During this process, two types of structures are produced: amphisomes, containing both endocytic and autophagic material, and autolysosomes, containing autophagic and lysosomal material such as hydrolases (Eskelinen 2005). There are three types of autophagic vacuoles (AVs) that are classified based on their morphology and enzymatic activity. Early autophagosomes, named AV-initial (AVi), are double-membrane structures with non-degraded cytoplasmic content; AV-intermediates (AVi/d) are single- or double-membrane amphisomes that show early signs of cargo degradation as well as small internal vesicles delivered by fusion of multivesicular endosomes. Lastly, AV-degradatives (AVd) are autolysosomes showing various levels of degradation. Two studies from 1990 addressed the stage at which the autophagic and endocytic pathways fuse with each other (Dunn 1990, Tooze et al. 1990). Dunn showed that in rat cells, AVs matured into degradative vacuoles in a stepwise fusion process with endolysosomal vesicles at different maturation stage; AV maturation occured via acquisition of lysosomal membrane proteins and lysosomal enzymes and subsequent acidification of the lumen (Dunn 1990). Tooze et al. used horseradish peroxidase (HRP) in their study and showed that this marker was first seen in amphisome-like structures indicating the merging of early endosomes and AVis (Tooze et al. 1990). Another study showed that in rat liver cells, AVis fuse with vesicular endosomes and multivesicular bodies (MVBs) (Liou et al. 1997). All these studies confirmed that the fusion between the autophagic and the endocytic pathways is a multi-stage process. The fusion between autophagic structures and late

endosomes/lysosomes is thought to occur mostly via a kiss-and-run fusion mechanism by which the content of a vesicle is transferred into the other vesicle while keeping the two vesicles incompletely fused (Jahreiss et al. 2008). After a short time, the two vesicles separate from each other.

1.6.2 Autophagosome-lysosome fusion machinery

Our current knowledge of the machinery involved in autophagosome-lysosome fusion is based on a general understanding of the membrane fusion machinery and intracellular trafficking. Several SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) and various membrane-tethering factors regulate the fusion of the autophagosome with late endosomes/lysosomes (LEs/lysosomes). Two independent SNARE complexes are known to be involved in these membrane fusion events (Figure 14) (Itakura et al. 2012, Matsui et al. 2018).

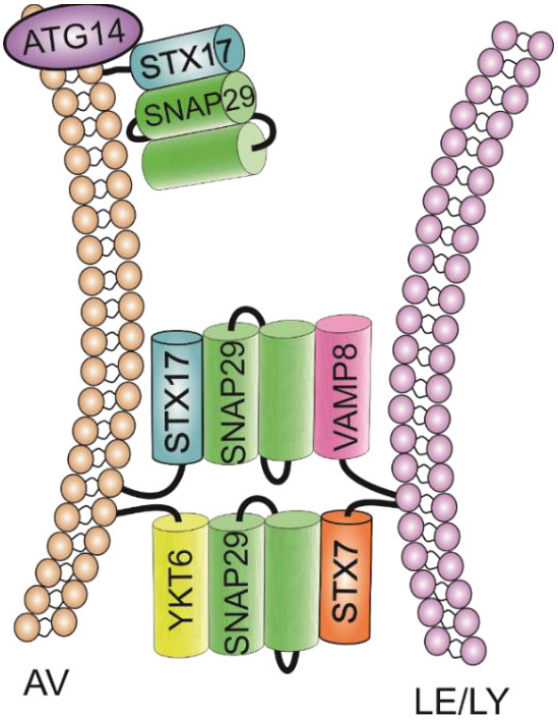

***Figure 14**: Schematic representation of the SNARE complexes involved in autophagosome-lysosome fusion events (see text for details).*

One complex contains the autophagosomal-localized STX17 (syntaxin 17), SNAP29 (synaptosomal nerve-associated protein 29) and the endosomal-localized VAMP8 (vesicle-associated membrane protein 8). The second complex consists of the autophagosomal SNARE protein YKT6, SNAP29 and lysosomal-resident protein STX7. STX17 is found on the outer membrane of closed autophagosomes while being absent from phagophore membranes. This is thought to inhibit the fusion of lysosomes with open autophagic structures. STX17 interacts with SNAP29 and VAMP8 to coordinate fusion events between autophagosomes and lysosomes. Its depletion causes autophagosome accumulation by inhibiting their degradation via lysosomal hydrolases (Itakura et al. 2012). A recent study showed that autophagosome-lysosome fusion is partially retained in STX17 knockout HeLa cells (Matsui et al. 2018). Accordingly, the SNARE protein YTK6 was shown to form a complex with SNAP29 and STX7, regulating autophagosome-lysosome fusion. The depletion of YKT6 partially inhibited autophagosome-lysosome fusion in wild-type cells but totally disrupted autophagosome-lysosome fusion in STX17 knockout cells.

Membrane-tethering factors enhance vesicle fusion efficiency by facilitating the docking and fusion steps of the opposing membranes and by stabilizing and stimulating the SNARE complexes. Several tethering factors that localize on autophagosomes and/or on late endosomes/lysosomes have been identified (Brocker et al. 2010). Rab7 is one of the most important tethering factors involved in the regulation of fusion events in autophagy (Gutierrez et al. 2004, Jager et al. 2004). Rab7, located on late endosomes/lysosomes, recruits other tethering factors such as EPG5, PLEKHM1 and HOPS and promotes the assembly of the SNARE complex for fusion evets. ATG14 is localized on autophagosomes and directly binds to the STX17-SNAP29 binary complex, stabilizing the complex and preparing it for VAMP8 interaction and autophagosome-endolysosome fusion (Diao et al. 2015). EPG5, a Rab7 effector, recognizes autophagosomes via direct interaction with LC3 and the STX17-SNAP29 dimer (Wang et al. 2016). EPG5 stabilizes the STX17-SNAP29-VAMP8 complex, coordinating fusion between autophagosomes and late endosomes/lyso-somes. PLEKHM1 (Pleckstrin homology domain containing protein family member 1), another Rab7 effector, is located on late endosomes/lysosomes and directly interacts with the HOPS complex as well as with LC3 via its LIR domain, docking autophago-some membranes to endosome/lysosome membranes and promoting the assembly of the SNARE complex (McEwan et al. 2015).

1.6.3 Movement of autophagosomes and lysosomes

Autophagosomes are generated throughout the cytoplasm while lysosomes are primarily concentrated in the perinuclear area of the cell (Jahreiss et al. 2008, Pu et al. 2016). In neuronal cells, autophagosomes often form in distal axons while most

lysosomes are located in the cell soma (Katsumata et al. 2010). Autophagosomes and lysosomes move bidirectionally within the cell in order to meet and take part in fusion events. Autophagosomes fuse with perinuclear-located lysosomes by travelling towards the nucleus or, alternatively, fuse with lysosomes moving towards the periphery. The movement towards the nucleus, also known as minus-end transport, is regulated by the motor protein dynein (Kimura et al. 2008), while movement to the opposite direction (plus-end transport) is controlled by the kinesin motors (Jahreiss et al. 2008, Pu et al. 2016). Fusion frequency as well as the recruitment of tethering factors are highly influenced by the cellular movement of autophagosomes, amphisomes and lysosomes. Dynein interacts with dynactin and forms a large complex that is essential for autophagosome movement towards the centrosome (Kimura et al. 2008). Abrogation of dynein activity causes impaired autophagosome-lysosome fusion and decreased autophagic clearance of aggregates, leading to motor neuron disease (Ravikumar et al. 2005). Moreover, inhibition of the dynein motor by EHNA (erythro-9-[3-(2-hydroxynonyl)] adenine) blocks autophagosome movement towards the soma in neuronal cells (Katsumata et al. 2010).

FYCO1, a Rab7 effector, interacts with LC3 and PI3P and mediates plus-end transport of autophagosomes in the cell (Pankiv et al. 2010). In the absence of the protein FYCO1, movement of autophagosomes is severely impaired leading to a perinuclear accumulation of autophagic structures.

In addition to the aforementioned factors, the BORC complex is needed for efficient autophagosome-lysosome fusion (Jia et al. 2017). The BORC complex interacts with ARL8 on lysosomes, enhancing their association with kinesin and promoting lysosomal transport towards the cell periphery. Depletion of the components of the BORC complex causes perinuclear clustering of lysosomes, reduced fusion events between autophagosomes and lysosomes and LC3-II accumulation due to inefficient autophagosome clearance. The BORC complex also promotes the assembly of the STX17-SNAP29-VAMP8 complex via ARL8-mediated recruitment of the HOPS complex (Jia et al. 2017). Therefore, the movement of autophagosomes and lysosomes not only affects the number of encounters between these organelles but also influences the recruitment of tethering factors as well as the assembly of the SNARE complexes involved in the fusion the process.

1.6.4 Autophagic lysosome reformation (ALR)

In recent years, several studies have unveiled a terminal step of autophagy also known as autophagic lysosome reformation (ALR) (Chen and Yu 2018). This process restores lysosomal availability after autophagy and is important for lysosomal homeostasis. After 4 hours of starvation, most lysosomes are consumed in normal rat

kidney cells (NRK) as one autophagosome can fuse with multiple lysosomes (Yu et al. 2010). After 12 hours of starvation, lysosomes recover back to a normal size and number. Lysosome recovery is based on the formation of tubular structures that extend from autolysosomes and are positive for LAMP1 and void of LC3 (Yu et al. 2010). Free LAMP1-positive vesicles were observed budding from the tip of these tubular structures. These autolysosome-originating vesicles, also known as proto-lysosomes, were initially nonacidic and lacked degradative capacity; proto-lysosomes then acquired degradative properties and matured into functional lysosomes (Yu et al. 2010). Interestingly, ALR is regulated by mTOR activity (Yu et al. 2010, Chen and Yu 2018). During prolonged starvation, nutrients generated by autophagy activated mTOR which was shown to induce ALR. Inhibition of mTOR activity induced autophagy, inhibited ALR and caused accumulation of enlarged autolysosomes (Yu et al. 2010).

Spinster (SPIN), a membrane protein found on late endosomes and lysosomes, is a sugar transporter that is also involved in ALR (Rong et al. 2011). Impaired Spinster function caused accumulation of giant autolysosomes after prolonged starvation, which suggested that the sugar transport function of Spinster was required for correct ALR. Other essential factors involved in ALR include clathrin, $PI(4,5)P_2$ (phosphatidylinositol-4,5-biphosphate), the $PI(4,5)P_2$-related kinase PIP5K1B and the kinesin motor protein KIF5B (Rong et al. 2012, Du et al. 2016). During ALR, PIP5K1B kinase converts PI4P to $PI(4,5)P_2$ and allows the accumulation of the latter on autolysosomes. $PI(4,5)P_2$ then recruits clathrin on autolysosomes via AP2 (adapter protein 2), inducing tubules formation (Rong et al. 2012). Clathrin forms small clathrin-enriched buds on autolysosome membranes. KIF5B drives autolysosome tubulation by pulling the clathrin-enriched autolysosomal membranes along microtubules (Du et al. 2016). Formation of proto-lysosomes from the tubule tips requires the GTPase DNM2/dynamin 2 which is thought to provide the mechanical force for vesicle scission (Schulze et al. 2013). Knockdown of PIP5K1A, another PI4P kinase, induces extensive autolysosome tubulation by blocking proto-lysosomes budding (Rong et al. 2012). Interestingly, also the PI3P generated by the Beclin 1/VPS34 UVRAG-containing complex plays a role in the proto-lysosome scission step in addition to its well-known function in autophagosome maturation (Munson et al. 2015). mTOR directly phosphorylates UVRAG on Ser550 and Ser571 and induces the kinase activity of VPS34. Abrogation of these phosphorylation sites causes an increase in the number and length of autolysosomal tubules and cell death (Munson et al. 2015).

1.7 Autophagy at cellular and organismal level

Autophagy is an adaptive process that is activated in response to various forms of stress including nutrients deprivation, infection and hypoxia. Basal autophagy is involved in protein and organelle turnover. Autophagy is important for cellular homeostasis and is rapidly upregulated during fasting or other cellular stresses in order to produce intracellular nutrients and building blocks for biosynthesis. Autophagy is an extremely important cytoprotective system as it also selectively degrades harmful cellular cargo such as damaged mitochondria and protein aggregates. Cardiomyocytes and neuronal cells are post-mitotic cells that highly depend on effective autophagy for quality control of proteins and organelles (Damme et al. 2015, Geronimo-Olvera and Massieu 2019, Yamaguchi 2019). The housekeeping function of autophagy is important for the viability of both cells and organisms. Autophagy defects are implicated in many diseases such as neuro-degeneration, cancer, infectious and metabolic diseases and immunological disorders (Dikic and Elazar 2018, Eskelinen 2019). Several studies have shown that functional autophagy is important for homeostasis in non-dividing cells as these cells are unable to dilute their cytoplasm by cell division that could counteract the accumulation of harmful cell stressors.

Knockout of autophagy genes has been used to study the *in vivo* functions of autophagy. Atg7 deficiency in liver cells caused various cellular aberrations such as deformed mitochondria, accumulation of peroxisomes, aberrant concentric membranous structures and ubiquitin-linked protein aggregates associated with cellular degeneration (Komatsu et al. 2005). Atg5 deletion in neuronal cells caused accumulation of cytoplasmic protein aggregates in inclusion bodies (Hara et al. 2006). This phenotype was linked to the appearance of neurodegeneration with behavioral defects, impaired movement and neuronal loss in the brain. These studies showed that autophagy is important for cellular homeostasis by the elimination of superfluous and damaged organelles that are likely to cause cellular distress and disease.

The aggregation of misfolded proteins and protein aggregates is one of the hallmarks of neurodegenerative diseases and plays a major role in the etiopathogenesis of diseases such as Alzheimer´s disease (AD), Parkinson´s disease (PD), Huntington´s disease (HD) and amyotropic lateral sclerosis (ALS) (Giacomelli et al. 2017). As mentioned before, neuronal cells that accumulate misfolded proteins are prone to defective cellular functions and cell death, and animals deficient in autophagy in neuronal cells show accumulation of ubiquitin-linked protein aggregates in neurons and die of neurodegenerative disorders (Komatsu et al. 2005). Several studies show

that autophagy plays a pivotal role in neurons by degrading and removing aggregate-prone proteins. The hallmark of Alzheimer´s disease is the presence of β-amyloid (Aβ) peptide-containing plaques and intraneuronal neurofibrillary tangles consisting of Tau proteins. Parkinson´s disease is characterized by the presence of intraneuronal inclusions known as Lewy bodies whose major component is the protein α-synuclein. Huntington´s disease is caused by expanded GAG trinucleotide repeats in the huntingtin gene. Due to the extended polyglutamine repeats, the Huntingtin protein is prone to aggregation and accumulates in the cell. The general hypothesis assuming that each neurodegenerative disease is linked to a single protein dysfunction has been revised through new results showing that various disease-related proteins can form toxic heteroaggregates by interacting with each other (Giacomelli et al. 2017). To date, there is still no curative therapy available for neurodegerative diseases. However, recent studies provide better insights into the mechanisms underlying neurodegeneration, enabling the discovery of new pharmacological targets and therapies (Ciechanover and Kwon 2015, Boland et al. 2018).

Autophagy also plays an important role in cancer, although its exact function is still under debate because autophagy activity is highly context-dependent in cancer cells (Dikic and Elazar 2018, Eskelinen 2019). The first link between autophagy and cancer was the discovery of the oncosuppressor gene beclin 1: the expression of beclin 1 was observed to be decreased in human breast, ovarian and prostate cancers. (Liang et al. 1999, Qu et al. 2003, Yue et al. 2003). In line with this, beclin $1^{+/-}$ mutant mice showed an increased incidence of tumors (Qu et al. 2003, Yue et al. 2003).

Autophagy plays an oncosuppressive role by preventing the accumulation of damaged or harmful organelles and proteins (White et al. 2015). Autophagy also acts to decrease metabolic and genomic stress (Karantza-Wadsworth et al. 2007). Selective degradation of damaged mitochondria reduces oxidative stress and chronic tissue damage, inhibiting pro-oncogenic signaling. In contrast to the preventive role of autophagy in tumor formation, it has been shown that several established cancers rely on autophagy for survival (Yun and Lee 2018). In this context, autophagy acquires tumor-promoting functions and supports the elevated nutrient demand of cancer cells, since energy and oxygen levels are low within the tumor microenvironment. The dual role of autophagy in cancers complicates the development of a reliable autophagy-based therapy for cancer. Clearly, several factors must be considered, such as the type, stage and genetics of the tumor microenvironment.

Caloric restriction (CR) is a well-known strategy for increasing life span and health span (Madeo et al. 2015). Dietary restriction is known to strongly induce the

autophagic response. The aging phenotype is due to the accumulation of malfunctioning organelles, defective proteins, aggregates and/or mutations in DNA. Autophagy counteracts the accumulation of these cellular stressors in order to support cellular fitness. Several genetic studies have shown that an extended life span is dependent on autophagy (Hansen et al. 2018, Nakamura and Yoshimori 2018, Pattison and Korolchuk 2018). For instance, overexpression of ATG5 in mice extends their average lifespan by 17.2 % (Pyo et al. 2013). ATG5 overexpression enhanced autophagy response and improved several aging-related traits such as leanness, insulin sensitivity and impaired movement.

The hippocampus is a key regulator of learning and memory in the human brain, and the formation of memory depends on hippocampus activity (Bartsch and Wulff 2015). Aging negatively affects the hippocampus leading to defective synaptic plasticity, impaired learning and poor memory. The decline in cognitive function is at least partially caused by the impairment of autophagy activity in the hippocampus (Glatigny et al. 2019). This study showed that stimulating memory formation induced autophagy in the hippocampus. Enhancing autophagy activity in the hippocampus also led to the formation of new memory through modulation of synaptic plasticity. Furthermore, young plasma containing osteocalcin induced autophagy in the hippocampus which rejuvenated memory in aged mice in an autophagy-dependent manner (Glatigny et al. 2019).

2 AIM OF THE STUDY

The aim of this doctoral thesis was to study autophagosome biogenesis in starvation induced and selective autophagy. The specific aims were as follows:

1: To study the roles of the protein TRIM17 in selective autophagy.

2: To further characterize the phenotype of the progressive neurodegenerative disease linked to a missense mutation in the cysteine proteinase gene ATG4D in Lagotto Romagnolo dogs.

3: To study the effects of the subcellular localization of Beclin 1 on autophagosome biogenesis.

3 MATERIAL AND METHODS

The table below presents a list of the materials and methods used in this doctoral dissertation (table 4). A minor contribution by the author in the listed methods is indicated by an asterisk (*). A lack of contribution by the author in the listed methods is indicated by a double asterisk (**). Methods used in the unpublished data are described below.

***Table 4**: List of materials and methods used in the doctoral dissertation*

Method	Publication
Cells	
HeLa	I, III
HEK293	I, III
HeLa-GFP-MKLP1**	I
Single cycle HIV-1 virus**	I
Dog primary fibroblasts*	II
Mouse embryonic fibroblast (MEF), wild type	III
MEF, ULK1/2 knock out	III
HEK293 Twin-StrepII-HA-Beclin 1	III
Animals	
ATG4D$^{mut/mut}$ Lagotto Romagnolo dogs (LR)**	II
ATG4$^{wt/wt}$ Lagotto Romagnolo dogs (LR)**	II
Molecular Biology	
Plasmid construction*	I, III
Quantitative RT-PCR**	I
PCR	I, III
Cell Biology	
Transfection	I, III
Construction of stable cell lines**	III
Virus infection**	I
siRNA silencing**	I
Flow cytometry**	I
Microscopy sample preparation	
Immunofluorescence	I, II, III
Epon embedding	I, II, III
Thin sectioning**	I, II, III
Immunohistochemistry**	II
Image acquisition	
Transmission electron microscopy	I, II, III
Laser scanning confocal microscopy	I, III
Wide-field fluorescence microscopy	II, III

***Table 4**: List of materials and methods used in the doctoral dissertation (continued)*

Method	Publication
Image acquisition	
Slidebook 6 software**	I
Cellomics HCS scanner**	I
Biochemistry	
Western blot	I
SDS-PAGE	III
BCA protein measurement assay	III
Thin-layer chromatography**	II
Lysosomal enzyme activity**	II
Co-immunoprecipitation**	I
Affinity purification (AP)**	III
Liquid chromatography-mass spectrometry (LC-MS)	III
Glycosaminoglycan (GAG) concentration**	II
Beta-galactosidase (b-Gal) activity**	II
Arylsulfatase A (ARYLS-A) activity**	II
Glycosylasparaginase (GA) activity**	II
Data processing	
SpotDetector BioApp**	I
Colocalization BioApp**	I
iDEV software**	I
BD FACScan**	I
ViiA 7 QuantStudio Software v1.2.4**	I
CellProfiler	II, III
ImageJ	II, III
Proteome Discoverer 1.4**	III
UniProtKB/Swiss Prot database**	III
SEQUEST**	III
Significance Analysis of INTeractome (SAINT)-express 3.6**	III
Quantitation	
Gene expression**	I
Western blot densitometric quantitation	I, II, III
Colocalization measurements	I, III
Spot counting*	I, II
Fluorescent intensity in vesicles per cell	III
Fluorescent intensity per cell	III

Quantification of mitochondrial distribution

Human cervical carcinoma (HeLa) cells were plated on glass coverslips. 24 h after seeding, cells were transfected with eGFP-Beclin 1-MITO and eGFP-MITO targeting control constructs using the jetPRIME transfection reagent (PolyPlus Transfection). 24 h after transfection, cells were starved with EBSS for 1 h and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline at room temperature for 30 min. Cells were permeabilized with 0.2% saponin in PBS for 10 minutes and blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes. Cells were labelled with rabbit anti-TOM20 (1:2000, Santa Cruz Biotechnology). After primary antibody incubation, cells were incubated with a secondary antibody conjugated to Alexa Fluor 594 (goat anti rabbit AF-594, Invitrogen, A-11037). Coverslips were mounted on microscope slides with Mowiol (Calbiochem) containing the antifading agent 1,4-Diazabicyclo [2.2.2] octane (DABCO, Sigma, D-2522). Images were taken with a fluorescent microscope (OLYMPUS AX70, PlanApo 60X/1,40 oil Corr/0,17).

The distribution of mitochondria, assessed using TOM20 labelling, was quantified in cells expressing the eGFP-Beclin 1-MITO and eGFP-MITO constructs by point counting. Fifteen cells per sample were imaged. A stereological test grid was created in order to count the area covered by mitochondria and cell cytoplasmic area. CorelDraw was used to superimpose the stereological grid onto the fluorescent image. Intersections of the points of the grid positive for mitochondria or cell cytoplasm were counted for each cell. Values obtained for mitochondria were divided by values obtained for cell cytoplasm in order to calculate the proportion of cell area covered by mitochondria.

4 RESULTS

4.1 Dual role of TRIM17 in selective autophagy (I)

TRIM proteins play an important role in selective degradation of cellular cargo. In a previous study, TRIM17 was shown to act as a bulk autophagy inhibitor while exhibiting biochemical features of autophagy-inducing TRIMs (Mandell et al. 2014). In this study, we demonstrated that TRIM17 inhibits selective degradation of a subclass of cellular cargos while inducing autophagic degradation of others.

4.1.1 TRIM17 inhibits selective degradation of part of autophagy targets

The study was initiated by testing how TRIM17 regulates the degradation of different known targets of selective autophagy. TRIM17 knockdown was achieved using siRNA, and it was observed to reduce the amount of p62 in HeLa cells, suggesting an inhibitory role for TRIM17 in autophagic degradation of p62 (I: Figure 1A-C). Total p62 protein levels and p62 puncta were reduced upon siRNAs silencing of TRIM17. Moreover, while p62 abundance was reduced in TRIM17 silenced HeLa cells, the protein level of LC3-II was not affected by the silencing (I: Figure 1D-F). Cells treated with Bafilomycin A1 to inhibit lysosomal protein degradation showed increased p62 protein levels, confirming negative regulation of p62 autophagic degradation by TRIM17. IFT20 and OFD1 are proteins involved in primary ciliogenesis and substrates for autophagic degradation (Pampliega and Cuervo 2016). Overexpression of GFP-TRIM17 increased IFT20 and OFD1 protein levels, while addition of Bafilomycin A1 had no additional effect, suggesting that TRIM17 negatively regulates the degradation of these proteins (I: Figure 1J). Moreover, the results also showed that TRIM17 itself was not an autophagy target (I: Figure 1I-J). Lastly, autophagic degradation of TRIM5α and the HIV-1 capsid protein p24 were also found to be inhibited by TRIM17 (I: Figure 2A-F).

Taken together, these data showed that TRIM17 inhibits selective autophagic degradation of various cellular targets.

4.1.2 TRIM17 is a platform for the assembly of Mcl-1-Beclin 1 complex

In order to understand the mechanism by which TRIM17 inhibits autophagy, the interaction between TRIM17 and Mcl-1 (myeloid cell leukemia-1) was studied. Mcl-1, a known interactor of TRIM17, is a Bcl-2 family protein that binds to the BH3 domain of Beclin 1, inhibiting autophagy (Erlich et al. 2007, Magiera et al. 2013). GFP-tagged TRIM17 and FLAG-tagged Beclin 1 were observed to colocalize in HeLa cells while over-expressed Mcl-1 did not show colocalization with TRIM17 (I: Figure 3D-E). HeLa

cells co-expressing TRIM17, Beclin 1 and Mcl-1 showed colocalization of these three proteins, suggesting that Beclin 1 recruits Mcl-1 and coordinates the formation of Beclin 1-Mcl-1 complexes on TRIM17 to inhibit autophagy (I: Figure 3F). In the absence of Mcl-1, cells expressing GFP-TRIM17 accumulated lower amounts of p62 compared to control cells, indicating that Mcl-1 activity is required for the inhibitory effects of TRIM17 in autophagy (I: Figure 4E).

Collectively, these results indicated that TRIM17 acts as a platform coordinating the formation of the Beclin 1-Mcl-1 complex that negatively regulates autophagy.

4.1.3 TRIM17 induces selective autophagy of midbodies

In order to find putative autophagy targets of TRIM17, TRIM17 was studied in connection with known autophagic proteins. GFP-tagged TRIM17 was found to colocalize with cellular structures positive for both p62, ubiquitin and LC3B (I: Figure 5A-B and Figure 6A). Moreover, correlative light-electron microscopy (CLEM) studies confirmed that GFP-tagged TRIM17 colocalized with aggregate-like structures (I: Figure 5C). The midbody ring (MR) was considered as a probable autophagy target of TRIM17. Midbody rings are protein-dense structures that are formed at the abscission site when two daughter cells are separated at the end of cytokinesis and that contain components of the cell division machinery (Chen et al. 2013). The midbody ring is transported to one of the two daughter cells and degraded by autophagy (Isakson et al. 2013). MKLP1 (mitotic kinesin-like protein 1) was used as a marker for midbody rings. GFP-tagged TRIM17 was observed to colocalize with endogenous MKLP1, and knockdown of TRIM17 induced midbody accumulation in the cells, suggesting a positive regulation of midbody degradation via TRIM17-dependent autophagy (I: Figure 6B-F).

To further investigate how TRIM17 inhibits selective degradation of certain autophagy targets while positively regulating midbody degradation, the role of Mcl-1 in autophagy of midbodies was investigated. Overexpressed Beclin 1 and TRIM17 both colocalized with the midbody marker MKLP1, while FLAG-tagged Mcl-1 did not colocalize with the latter, suggesting that TRIM17 complexes lacking Mcl-1 induced autophagic degradation of midbodies (I: Figure 7B-C). Additional TRIM proteins (TRIM21, TRIM47 and TRIM76) were also identified that contributed, alongside TRIM17, to selective autophagy of midbodies (I: Figure 8).

To summarize, this study demonstrated that TRIM17 inhibits selective autophagy of various known targets and at the same time positively regulates autophagic degradation of midbodies. The protein Mcl-1 plays an essential role in deciding whether TRIM17 will perform autophagy-inducing or -inhibiting functions in the cell.

TRIM17 inhibits autophagy by allowing the formation of the Beclin 1-Mcl-1 complex; when Mcl-1 disassociates from Beclin 1, TRIM17 can perform its pro-autophagic activity involving the degradation of cellular midbodies.

4.2 ATG4D is needed for basal autophagy (II)

Previous studies by Pernilla Syrjä and colleagues showed that a missense mutation (c.1288G>A) in the gene encoding the protein ATG4D is linked to a progressive neurological disease in Lagotto Romagnolo dogs (LRs) (Kyöstilä et al. 2015). Tissues of the affected dogs showed severe neuronal cytoplasmic vacuolization and impaired autophagy flux. The aim of this study was to further investigate how the ATG4D mutation affected autophagy in LR dogs. Moreover, the histopathology of affected tissues was further characterized, and the affected dogs were screened for known symptoms of lysosomal storage diseases.

4.2.1 ATG4D mutation alters basal autophagy

In order to study how autophagy was affected by the ATG4D missense mutation, we first studied endogenous LC3-II protein levels by immunoblotting. In nutrient-rich conditions, LC3-II levels were significantly higher in fibroblasts isolated from affected dogs compared to cells from control animals (II: Figure 1A-B). Upon addition of Bafilomycin A1 in nutrient-rich conditions, the LC3-II levels increased in both samples, but the increase was smaller in affected cells compared to control cells, suggesting a possible defect in the lysosomal degradation of LC3-II in cells carrying the ATG4D mutation (II: Figure 1A-B). In starved cells, LC3-II amounts were comparable between the control and affected cells (with or without addition of Bafilomycin A1) (II: Figure 1A-B). These results indicated that the ATG4D mutation affects basal autophagy and not starvation-induced autophagy. Similar results were obtained by quantifying LC3-positive spots, assumed to represent autophagosomes, in immunofluorescent microscopy images (II: Figure 2A-I).

Together, these results indicated that the ATG4D mutation causes a defect in basal autophagy but did not impair starvation-induced autophagy. Moreover, our results suggested a defect in the lysosomal degradation of autophagic cargo in affected cells.

4.2.2 Ultrastructural analysis of affected cells shows abnormal vacuolization

The ultrastructure and morphology of affected tissues was also assessed. Aberrant cytoplasmic vacuolization was observed in various affected tissues including the apocrine sweat glands of the skin, renal glomerula and plasma cells of the lymph nodes (II: Figure 3-6). Vacuoles in sweat glands showed clear LAMP2-positive

staining while the smooth muscle cells showed LC3B- and p62-positive cytoplasmic granules in immunohistochemical staining (II: Figure 7-8). Electron microscopy studies showed numerous single-membrane vacuoles within the cytoplasm of affected tissues (II: Figure 9-14). The vacuoles showed no electron-dense content. Sporadically engulfment and fusion events between two vacuoles or between autophagic structures and vacuoles was observed (II: Figure 9-10,12, arrows).

4.2.3 ATG4D mutation is not linked to lysosomal storage diseases

The ultrastructural studies indicated a possible link between the ATG4D mutation and the occurrence of lysosomal storage diseases in the affected dogs. In order to investigate this hypothesis, biochemical screening of affected dogs was performed to find possible indicators of lysosomal storage disease. Affected and control dogs showed comparable levels of excreted oligosaccharides and sialic acid in their urine. Affected dogs did not show any extra bands in thin-layer chromatography of oligosaccharides, indicating normal excretion patterns and the absence of disease markers (II: Figure 1S). In order to confirm these results, the activity of selected lysosomal hydrolases was measured in cultured dog fibroblasts and the culture medium (II: Table 3). Affected cells showed a slight increase in intracellular enzymatic activity compared to control cells while extracellular enzymatic activity was comparable between affected and control cells (II: Table 3). Taken together, these results indicate that lysosomal degradation is mildly altered in affected dogs as the cellular activity of the three lysosomal enzymes was increased in affected dogs.

To summarize, this study showed that LR dogs carrying the ATG4D missense mutation present defective basal autophagy together with abnormal cytoplasmic accumulation of single-membrane vesicles. Cells from affected dogs showed higher LC3-II protein levels as well as more LC3 puncta in nutrient-rich conditions compared to cells from control dogs, while the difference was not detected in starved cells. Moreover, the results excluded impaired lysosomal degradation in the affected dogs.

4.3 Beclin 1 localization in autophagosome biogenesis (III)

The origin of phagophore membranes still holds many unanswered questions, and various subcellular compartments such as the plasma membrane, ER, mitochondria and ER-Golgi intermediate have been implicated in autophagosome biogenesis (Hailey et al. 2010, Ravikumar et al. 2010, Orsi et al. 2012, Hamasaki et al. 2013). The Beclin 1/VPS34 complex plays an essential role in autophagosome biogenesis because it produces PI3P that is essential for the downstream recruitment of other autophagy effectors. In this study, we investigated how the subcellular localization of Beclin 1 affects autophagosome biogenesis. Moreover, we also studied if forced

Beclin 1 targeting to ER was able to rescue autophagosome formation in the absence of the ULK1 and ULK2 kinases.

4.3.1 Characterization of Beclin 1 constructs

To study the role of Beclin 1 in autophagosome biogenesis, we generated N-terminally epitope-tagged Beclin 1 containing a C-terminus targeting sequence to the ER and mitochondria (III: Figure 1A). We generated two different sets of Beclin 1 constructs, one containing an eGFP tag and another containing a Twin-StrepII-HA tag. We first tested the subcellular localization of Beclin 1 constructs in stable and inducible HEK293 cells expressing Twin-StrepII-HA-tagged Beclin 1 targeted to ER and mitochondria. We performed immunofluorescence against the ER markers BAP31 and calreticulin and against the mitochondrial marker TOM20. ER- and mitochondrial-targeted Beclin 1 constructs localized to their expected subcellular compartments (III: Figure 1B-C). Importantly, the stable expression of Beclin 1 targeted constructs did not alter ER or mitochondria morphology in HEK293 cells. We also studied the subcellular localization of the wild-type Beclin 1 construct in stable and inducible HEK293 cells expressing Twin-StrepII-HA-tagged Beclin 1-WT. Wild-type Beclin 1 showed predominantly diffuse cytoplasmic localization (III: Figure S1A-C).

We also used eGFP-tagged Beclin 1 constructs which were transiently transfected in MEF cells. ER-targeted Beclin 1 localized to the expected subcellular compartment as shown by immunofluorescence against the ER markers BAP31 and Calreticulin in MEF-WT cells expressing eGFP-tagged Beclin 1-ER (III: Figure 2A, S2A). ER-targeted eGFP-tagged Beclin 1 showed less colocalization with the soluble ER protein disulphide isomerase (PDI) and no colocalization with the Golgi marker GM130 (III: Figure S2B-C). We obtained similar results for the ER-targeted control construct containing no Beclin 1 sequence (III: Figure 2A, S2A-C). Since transient expression of Beclin 1 targeted to the ER induced morphological changes in the ER in MEF (III: Figure 2A), we performed CLEM in order to study ER morphology at high magnification (III: Figure 3A-D). Our results showed that the ultrastructure of the rough ER was normal in MEF-WT cells expressing ER-targeted eGFP-tagged Beclin 1. We did not observe any morphological changes in rough ER compared to non-expressing cells. We further investigated if targeting Beclin 1 to the ER would cause ER stress in order to make sure that the results on autophagosome formation were specifically caused by the targeting of the Beclin 1 construct itself and were not a side effect mediated by ER stress which is known to induce autophagy. We performed immunofluorescence against CHOP (CCAAT-enhancer-binding protein homologous protein), known to translocate to the nucleus upon ER stress. Tunicamycin, a

compound known to induce ER stress by inhibiting N-glycosylation, was used as positive control. Our results showed that targeting of Beclin 1 to the ER did not cause ER stress as demonstrated by the absence of CHOP nuclear staining in MEF-WT cells expressing the eGFP-tagged Beclin 1-ER construct (III: Figure S3). Moreover, the expression of the ER-targeted control and non-targeted Beclin 1 constructs also did not cause any ER stress in MEF-WT cells, while the tunicamycin treatment caused CHOP nuclear staining (III: Figure S3). Taken together, these results showed the correct subcellular localization of ER-targeted Beclin 1 and confirmed that targeting Beclin 1 to the ER did not affect ER ultrastructural morphology and did not cause ER stress.

Mitochondrially-targeted eGFP-tagged Beclin 1 and the mitochondrially-targeted control constructs both predominantly localized to the mitochondria (III: Figure 2B). Transient expression of Beclin 1 targeted to mitochondria induced strong changes in the distribution of mitochondria in MEF-WT cells as mitochondria were seen to accumulate in the perinuclear area (III: Figure 2B). We performed immunostaining against TOM20 in HeLa cells expressing the eGFP-tagged-Beclin 1 targeted to mitochondria and the eGFP-tagged mitochondrially-targeted control construct and quantified the cell area covered by mitochondria by point counting mitochondria in immunofluorescent images (Figure 15).

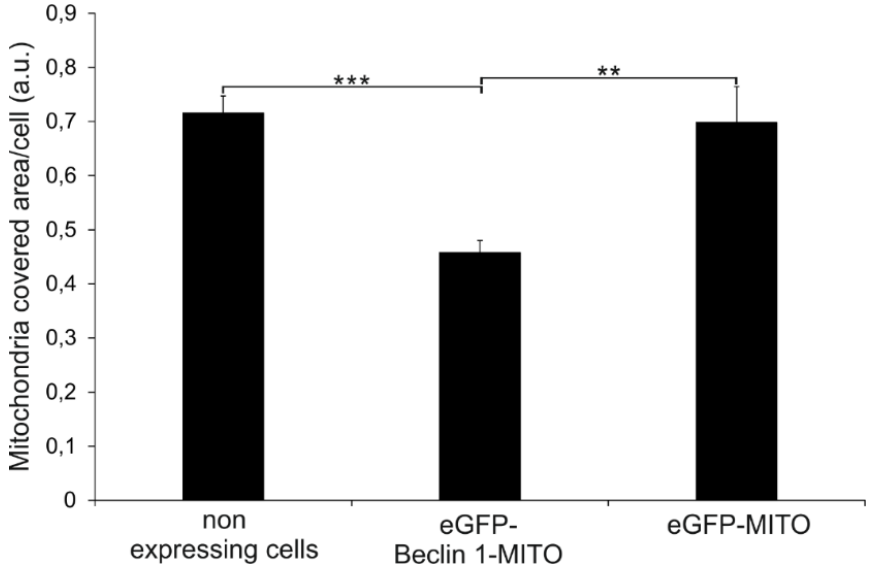

Figure 15*: Cell area covered by mitochondria. HeLa cells were transfected with eGFP-Beclin 1-MITO and eGFP-MITO constructs, starved for 1 hour and labelled against the mitochondrial marker TOM20. Point counting was performed to estimate the proportion of cell area covered by mitochondria. Results are shown as mean and SEM of 15 cells. One-way ANOVA followed by the Tukey Kramer post hoc test was used to test statistical significance. ** p < 0.01, *** p < 0.001*

Our results showed that mitochondria covered 45.8 % of the cell area in cells expressing eGFP-tagged Beclin 1 targeted to mitochondria while cells expressing the mitochondrially-targeted eGFP-tagged control construct and non-expressing cells showed similar values for areas covered by mitochondria (69.8 % and 71.6 % respectively).

We also used electron microscopy imaging in order to assess the morphology of mitochondria in MEF-WT cells expressing eGFP-tagged Beclin 1 targeted to mitochondria (III: Figure 3E-H). Our analysis showed that the ultrastructure of mitochondria was normal despite their clustering in the perinuclear area. As for the ER targeting, we also investigated possible mitochondrial stress caused by Beclin 1 forced targeting to mitochondria. We first used western blotting against OPA1 in order to monitor its cleavage in cells stably expressing Beclin 1 targeted to mitochondria. As positive control, mitochondrial stress was caused by the uncoupling drug CCCP (III: Figure S5). The lack of OPA1 cleavage confirmed that targeting of Beclin 1 to

mitochondria did not cause any mitochondrial stress in stable and inducible HEK293 cells expressing Twin-StrepII-HA-tagged mitochondrial-targeted Beclin 1. As expected, cells treated with CCCP displayed prominent OPA1 cleavage (III: Figure S5). These results were confirmed by quantification of the staining with the mitochondrial membrane potential indicator dye TMRE (tetramethylrhodamine, ethyl ester) in live cell imaging experiments in MEF-WT cells (III: Figure S4). As expected, cells expressing the eGFP-tagged Beclin 1 constructs, mitochondrially-targeted and non-targeted, as well as non-expressing cells, all had similar membrane potential. However, CCCP dramatically decreased mitochondrial membrane potential. The results confirmed that targeting of Beclin 1 to mitochondria did not cause any mitochondrial stress. Together, these results showed the expected subcellular localization of mitochondrially-targeted Beclin 1 and indicated that targeting Beclin 1 to mitochondria did not alter mitochondrial ultrastructure and did not induce mitochondrial stress.

To summarize these first findings, targeting of Beclin 1 to mitochondria or ER was successful as shown by the correct localization of Beclin 1 to the corresponding subcellular organelles. Moreover, both ER and mitochondria displayed normal morphology upon targeting of Beclin 1, and Beclin 1 targeting caused no stress to the corresponding organelles.

4.3.2 Interactions of targeted Beclin 1 and the effects of the constructs on autophagosome formation

In order to identify the binding partners of targeted Beclin 1 constructs, the stable and inducible HEK293 cell lines expressing the Beclin 1 constructs were used to perform affinity-purification of Beclin 1, followed by mass spectrometry (AP-MS) (III: Figure 4). The analysis revealed high-confidence interacting proteins (HCIP) for the Beclin 1 constructs under both nutrient-rich and starvation conditions. All Beclin 1 constructs made abundant interactions with the lipid kinase VPS34, the effector kinase VPS15 and UVRAG. These interactions were similar under both conditions. Non-targeted Beclin 1 made abundant interactions with ATG14, NRBF2 and Rubicon while ER-targeted and mitochondrially-targeted Beclin 1 made less abundant but still detectable interactions with them under both conditions. Interestingly, no AMBRA1 interactions were detected in our study (III: Figure 4).

To summarize, the results showed that all the Beclin 1 constructs interacted with all known autophagy-related Beclin 1 binding partners under both nutrient-rich and starvation conditions.

In order to study the effects of Beclin 1 targeting on autophagosome formation, we used stable and inducible HEK293 cell lines expressing Beclin 1 constructs and performed western blotting against LC3-II (III: figure 5A-D) and the autophagy cargo protein p62 (III: Figure S7A-B). Stable expression of all Beclin 1 constructs increased LC3-II protein levels compared to the parental cell line, in both full medium and starvation (III: Figure 5A-B). There were no statistically significant differences between the cell lines in the relative increase of LC3-II under starvation (LC3-II starvation/nutrient-rich) indicating that autophagosome formation was not affected by the targeting of Beclin 1 (III: Figure 5C). Moreover, only non-targeted Beclin 1 was able to slightly increase autophagy flux (LC3-II starvation+Bafilomycin A1/starvation) compared to the parental cell line and cells expressing the targeted Beclin 1 constructs (III: Figure 5D). However, the difference did not reach statistical significance. Western blotting against p62 revealed that none of the Beclin 1 constructs significantly altered p62 protein levels compared to parental cells (III: Figure S7).

Taken together, these findings indicated that all Beclin 1 constructs used were found in complex with all known autophagy-related Beclin 1 interactors except AMBRA1, and that none of them altered autophagosome formation or autophagy flux when compared to the parental cell line.

4.3.3 Effects of the Beclin 1 constructs on autophagosome formation in the absence of ULK1 and ULK2 kinases

We next investigated whether forced targeting of Beclin 1 could rescue autophagosome formation in ULK1 and ULK2 double knockout MEF cells. Previously, it was shown that in the absence of the ULK kinases autophagosome biogenesis is severely impaired (McAlpine et al. 2013). We used immunofluorescence staining of endogenous LC3 in order to follow autophagosome formation in the presence or absence of ULK1 and ULK2 kinases in MEF cells under nutrient-rich and starvation conditions with or without Bafilomycin A1 (Figure 16 and III: Figure 6, S8-12). Instead of using simply the count of LC3-positive puncta per cell, we quantified the proportion of LC3 signal originating from LC3 puncta, since we found that this value better reflected the known autophagy induction under starvation conditions.

First, we characterized the effects of the Beclin 1 constructs in wild type MEF. Expression of the non-targeted eGFP-tagged Beclin 1 in MEF-WT cells significantly increased the amount of LC3 in vesicles compared to non-expressing cells in all three conditions (Figure 16).

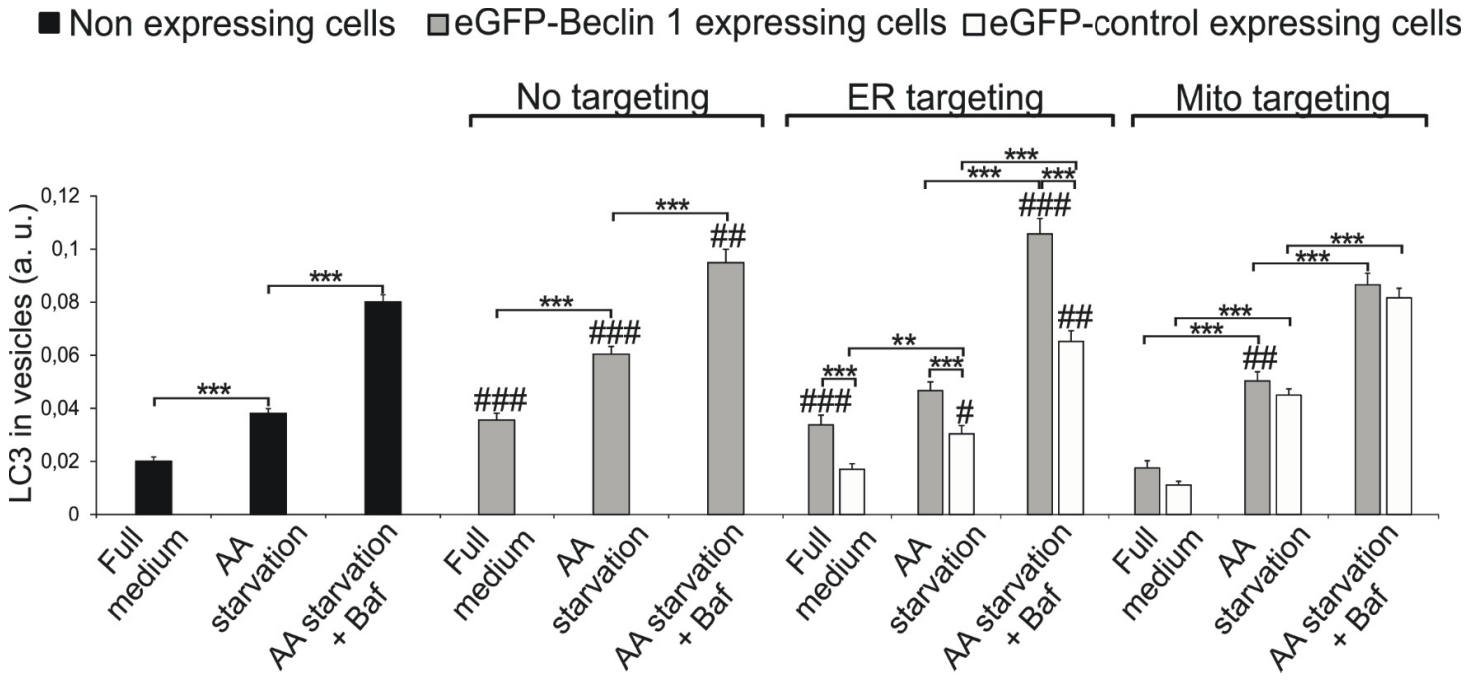

Figure 16: *Quantification of LC3 labelling in MEF-WT cells expressing the eGFP-tagged Beclin 1 constructs. MEF-WT cells were transfected with eGFP-Beclin 1-WT, eGFP-Beclin 1-ER, eGFP-ER, eGFP-Beclin 1-MITO, or eGFP-MITO. Cells were kept in full medium or starved in amino-acid (AA) free medium for 1 h with or without 100 nM Bafilomycin A1, fixed and labelled with LC3 antibody. CellProfiler was used to quantify the proportion of LC3 in vesicles, both in cells expressing the eGFP-tagged constructs and in non-expressing cells in the same sample. Results are shown as mean and SEM of 50 cells expressing eGFP-tagged construct and 150 non expressing cells (N = 50 and N = 150, respectively). One-way ANOVA followed by Tukey Kramer post hoc test was used to test statistical significance. ** $p < 0.01$, *** $p < 0.001$ for comparisons within cell lines. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ for comparisons between cells expressing eGFP-tagged constructs and non-expressing MEF-WT cells under the same condition. (a. u. arbitrary unit).*

As expected, in non-expressing cells amino acid starvation significantly increased vesicular LC3, and the addition of Bafilomycin A1 to the starvation medium further increased the amount. Similar results were observed in cells expressing non-targeted eGFP-tagged Beclin 1, and the overexpression of Beclin 1 increased the amount of vesicular LC3 under all conditions. ER-targeted eGFP-tagged Beclin 1 also signify-cantly increased the amount of LC3 in vesicles compared to non-expressing cells under nutrient-rich conditions. Upon starvation, non-expressing cells and ER-targeted eGFP-tagged Beclin 1 expressing cells had comparable amounts of LC3 in vesicles. In cells expressing eGFP-tagged Beclin 1-ER, the addition of Bafilomycin A1 significantly increased the amounts of LC3 in vesicles compared to non-expressing cells (Figure 16). Upon starvation, eGFP-tagged Beclin 1 targeted to mitochondria significantly increased the amount of LC3 in vesicles compared to non-expressing cells. Compared with non-expressing cells, the targeting controls eGFP-ER and eGFP-MITO had no effect on the amount of LC3 in vesicles, with the exception of eGFP-ER inducing a small decrease under starvation (Figure 16).

Taken together, the results in wild type MEFs showed that the quantification of vesicular LC3 was suitable for monitoring autophagy induction and that, in the wild type background, targeting Beclin 1 to ER or mitochondria only slightly affected autophagy.

We next studied the effects of the Beclin 1 constructs in MEF cells lacking ULK1 and ULK2 kinases. Upon amino acid starvation, expression of the non-targeted eGFP-tagged Beclin 1 construct in MEF-ULK1/2-KO cells significantly increased the amount of LC3 in vesicles compared to non-expressing cells (III: Figure 6, S9). Cells expressing the eGFP-tagged Beclin 1-WT construct accumulated similar amounts of LC3 in vesicles as MEF-WT upon starvation (III: Figure 6). The addition of Bafilomycin A1 to the starvation medium only mildly increased the amount of LC3 in vesicles in MEF-ULK1/2-KO cells compared to MEF-WT, indicating an impaired autophagy flux in ULK1/2-KO cells (III: Figure 6, S9).

We then studied the effects of ER-targeted Beclin 1 in MEF-ULK1/2-KO cells (III: Figure 6, S10A, S11). Compared to non-expressing cells, the expression of eGFP-tagged Beclin 1-ER significantly increased the amount of LC3 in vesicles both under nutrient-rich and starvation conditions (III: Figure 6, S10A, S11). Compared to MEF-WT cells, MEF-ULK1/2-KO cells expressing eGFP-tagged Beclin 1-ER accumulated significantly higher amounts of LC3 in vesicles under both conditions. However, the addition of Bafilomycin A1 did not significantly increase the amount of LC3 in vesicles indicating that, similar to non-targeted Beclin 1, also ER-targeted eGFP-tagged Beclin 1-induced autophagosomes could not flux in ULK1/2-KO cells (III: Figure 6, S10A, S11). Importantly, MEF-ULK1/2-KO cells expressing the targeting control eGFP-ER

showed similar amounts of LC3 in vesicles to non-expressing MEF-ULK1/2-KO cells, indicating that the targeting control had no effect on autophagosome formation (III: Figure 6, S10A, S11).

Lastly, we studied the effect of mitochondrially-targeted Beclin 1 in MEF-ULK1/2-KO cells (III: Figure 6, S10B, S12). eGFP-tagged Beclin 1 targeted to mitochondria showed similar effects to non-targeted eGFP-tagged Beclin 1 in MEF-ULK1/2-KO cells under starvation conditions (III: Figure 6, S10B, S12). The addition of Bafilomycin A1 to the starvation medium significantly increased the amounts of LC3 in vesicles MEF-ULK1/2-KO cells (III: Figure 6, S10B, S12) but the increase was not as prominent as in MEF-WT cells, indicating that autophagosomes formed in ULK1/2-KO cells could only partially flux (III: Figure 6, S10B, S12). MEF-ULK1/2-KO cells expressing the targeting control eGFP-MITO showed similar amounts of LC3 in vesicles to non-expressing MEF-ULK1/2-KO cells indicating that, similar to the ER-targeted control, the mitochondrially targeting control had no effect on autophagosome formation (III: Figure 6, S10B, S12).

We confirmed that the expression of Beclin 1 constructs in ULK1/2 knockout cells was able to induce autophagosome formation by performing CLEM (III: Figure 7-8). MEF-ULK1/2-KO cells expressing non-targeted, ER-targeted or mitochondrially-targeted Beclin 1 all contained autophagic structures with normal morphology, indicating that Beclin 1 can support autophagosome formation in the absence of ULK1 and ULK2 kinases (III: Figure 7-8).

Together, these results showed that Beclin 1 can partially rescue autophagosome formation in the absence of ULK1 and ULK2 kinases. Beclin 1 targeted to ER was the most effective in inducing autophagosome formation in cells lacking ULK1 and ULK2 kinases compared to non-targeted and mitochondrially-targeted Beclin 1. Moreover, autophagy flux was impaired in these cells, further indicating that Beclin 1 could not rescue autophagosome maturation in cells lacking ULK1 and ULK2 kinases. Finally, we demonstrated that ULK1 and ULK2 kinases regulate Beclin 1 localization at the ER under nutrient-rich conditions.

4.3.4 Localization of Beclin 1 during autophagy induction

Our results suggested that targeting Beclin 1 to the ER favours autophagosome formation. This raised the question whether Beclin 1 is enriched in the ER during autophagy induction. Thus, we studied the localization of wild type Beclin 1 during autophagy induction and the role of the ULK kinases in this process. We first used HEK293 cells inducibly expressing Twin-StrepII-HA-tagged wild type Beclin 1 and showed, using immunofluorescence staining, that upon starvation, Beclin 1 is signify-

cantly enriched in the ER (III: Figure 9A, S13). We then used MEF-WT and MEF-ULK1/2-KO cells expressing eGFP-tagged Beclin 1-WT to investigate whether ULK1 and ULK2 kinases play a role in Beclin 1 enrichment in the ER (III: Figure 9B, S14). In both wild type and ULK1/2-KO MEFs, starvation increased the colocalization of Beclin 1 and the ER marker BAP31. Non-starved MEF ULK1/2-KO cells showed significantly lower colocalization of Beclin 1 with the ER marker but, upon starvation, the colocalization was similar in both cell lines (III: Figure 9B, S14). These results suggested that, under basal conditions, the ULK kinases may play a role in maintaining a pool of Beclin 1 in the ER.

To summarize, we demonstrated that Beclin 1 is enriched in the ER during autophagy induction, and that ULK1 and ULK2 kinases regulate Beclin 1 localization at the ER under nutrient-rich conditions.

5 DISCUSSION

5.1 TRIM17 has a dual role in selective autophagy

Article I showed that TRIM17 inhibits autophagic degradation of several cellular targets while inducing selective degradation of midbodies. Moreover, the results showed that the dual activity of TRIM17 is regulated by the Mcl-1 protein. The assembly of the TRIM17-Beclin 1-Mcl-1 complex leads to autophagy inhibition while the formation of the TRIM17-Beclin 1 complex without Mcl-1 induces the selective autophagy of midbodies.

Midbody rings are organelles formed in the final steps of cytokinesis (Chen et al. 2013). They form at the abscission site, contain components of the cell division machinery and are inherited by one of the two daughter cells. It has been shown that midbodies can regulate several cellular functions such as cell polarity and cellular communication (Dionne et al. 2015). Moreover, midbodies can enhance the pluripotency of stem cells and increase the tumorigenic properties of cancer cells (Antanaviciute et al. 2018). Several studies have shown that midbodies are actively degraded via autophagy and that the absence of autophagy proteins or receptors can lead to the accumulation of midbodies (Kuo et al. 2011, Isakson et al. 2013, Dionne et al. 2017).

A siRNA screen in article I revealed three additional TRIMs (TRIM21, TRIM47 and TRIM76) involved in the autophagic degradation of midbodies in addition to TRIM17. Other studies have shown that TRIM21 and TRIM76 play a role in autophagy. TRIM21 acts as an autophagy receptor by recognizing IRF3 (interferon regulatory factor 3) and targeting it for degradation via autophagy (Kimura et al. 2017). Moreover, TRIM21-mediated ubiquitination regulates IKK-β protein levels via autophagic degradation of active IKK-β (Niida et al. 2010). TRIM76 was linked to autophagy in a previous study where several TRIMs were screened to assess their modulation of autophagy (Mandell et al. 2014). TRIM47 has been shown to be involved in cancer progression (Han et al. 2017, Liang et al. 2019) but its role in autophagy is still unknown.

Article I further explored the mechanism by which the autophagy machinery selectively “chooses” what to degrade by introducing the concept of autophagy machinery selectively “deciding what not to degrade”. The results showed that TRIM17 inhibited precision autophagy regulated by other TRIMs such as TRIM5α-mediated degradation of HIV-1 capsids. This expands the autophagy regulatory roles of TRIM17 not only to its direct substrates but also to substrates of other TRIMs.

TRIM28 also plays a dual role in autophagy regulation. TRIM28 promotes autophagy (Yang et al. 2013, Peng et al. 2019) and simultaneously downregulates the autophagic response via degradation of the protein kinase AMPK (Pineda and Potts 2015). Interestingly, a recent study shed light on TRIM17-TRIM28 combined regulation of ubiquitination of the protein BCL2A1 (Lionnard et al. 2019). TRIM28 acts as an E3 ubiquitin ligase for BCL2A1 while TRIM17 regulates BCL2A1 protein levels by inhibiting TRIM28-mediated ubiquitination of BCL2A1, regulating its stability and modulating cell death. This study further confirms that two different TRIMs can regulate common cellular targets.

In conclusion, article I showed that the dual role of TRIM17 is important in the regulation of cellular homeostasis by selective autophagy. The different properties embedded within each TRIM protein are important for enhancing precise cargo selection and efficient degradation via autophagy.

5.2 ATG4D plays a role in basal autophagy

In this study, we described a progressive neurological disease linked to a missense mutation in the ATG4D gene in LR dogs. Affected cells exhibited altered basal autophagy while prominent neuronal cytoplasmic vacuolization was seen in several tissues. Under nutrient-rich conditions, we showed increased levels of the protein LC3-II and LC3 spots in cells with the ATG4D mutation compared to control cells. Upon starvation, control and affected cells showed no difference, suggesting a defect in autophagy flux under basal conditions. The accumulation of LC3-II in the cells can be due to increased autophagosome formation or defective autophagic flux caused by the impairment of the autophagosome-lysosome fusion, reduced autophagic degradation, or both (Mizushima et al. 2008). When we added Bafilomycin A1 to the cells under nutrient-rich conditions, affected cells showed a milder increase in LC3-II protein levels compared to control cells, indicating that the basal autophagy flux was decreased in the affected cells. Since autophagy flux was not altered under starvation conditions, and we did not find evidence for lysosomal storage diseases, we postulated that autophagy flux was decreased due to impaired autophagosome-endosome/lysosome fusion and not defective lysosomal degradation.

Ultrastructural analysis of affected tissues showed abnormal accumulation of single-membrane vesicles in several tissues. These vesicles resembled enlarged endosomes, MVBs or amphisomes, suggesting an involvement of the endosomal pathway in the etiopathology of the disease. Other studies have shown a connection between basal autophagy and endo-lysosomal trafficking (Murrow and Debnath 2015, Murrow et al. 2015, Murrow and Debnath 2018). Cells lacking ATG12-ATG3

conjugation show defective basal autophagy and aberrant perinuclear accumulation of late endosomes and MVBs caused by impaired endo-lysosomal trafficking. Moreover, ATG12-ATG3 interact with the ESCRT-associated protein Pdcd6ip (programmed cell death 6 interacting protein, also known as Alix), and this interaction may mediate the regulation of basal autophagy, late endosome distribution, exosome biogenesis and viral budding (Murrow et al. 2015). Interestingly, starvation-induced autophagy was not altered in the absence of ATG12-ATG3. Another study showed that RAB24 was needed for the clearance of autophagic structures under nutrient-rich conditions but not during starvation (Yla-Anttila et al. 2015). Thus, several studies confirm that the molecular requirements of basal autophagy differ from those of induced autophagy.

We observed abnormal vesicular accumulation in various cell types in the affected dogs. A similar phenotype was previously described in human erythroid cells expressing dominant-negative mutants of ATG4B or ATG4D (Betin et al. 2013). Autophagy regulates organelle removal during erythroid differentiation. Cells expressing ATG4B or ATG4D mutants showed an increased accumulation of enlarged amphisomes. Interestingly, differentiation of erythroid cells was not disrupted by the accumulation of amphisomes, indicating that autophagy was functional despite the ultrastructural changes (Betin et al. 2013). These findings are in agreement with our study that did not detect alterations in the function of extraneural organs in LR affected dogs. The sole clinical phenotype the affected dogs exhibited were lesions in the Purkinje cells and neurons. Notably, Purkinje cells and neurons have previously been shown to be dependent on basal autophagy (Hara et al. 2006). A more recent study showed that defects in ATG4.2 in *Caenorhabditis elegans* alters autophagosome maturation and causes abnormal accumulation of multilamellar autophagosome-like vacuoles in neurons (Hill et al. 2019).

In addition to phagophores and autophagosomes, autophagy proteins can localize in single-membrane vacuoles in various cell types (Florey et al. 2011, Florey and Overholtzer 2012). LC3 is recruited to entotic vacuoles, to phagosomes engulfing apoptotic cells and to macropinosomes. This recruitment is dependent on ATG5, ATG7 and VPS34 activity (Florey et al. 2011). Moreover, LC3-II delipidation via ATG4 needs to be tightly regulated as it provides a pool of free LC3-I for the next conjugation round and prevents the accumulation of LC3-II on non-autophagic structures such as endosomes (Nakatogawa et al. 2012). It is possible that the phenotype observed in the affected LR dogs could be explained by disproportionate amounts of LC3-II in the cells, as well as incorrect deposition of LC3-II on endosomal membranes.
The cytoplasmic vacuolization seen in our study was prominent in cells active in secretion. Autophagy plays an important role in these cells: secretory granules are selectively degraded via autophagy in a process known as crinophagy, in which

secretory granules can directly fuse with lysosomes (Thachil et al. 2012). Our results did not indicate any defects in crinophagy in affected LR dogs. Salivary glands and pancreatic acinar cells did not accumulate secretory granules. Instead, the cytoplasmic vacuoles were single membrane-bound and there was no LC3B staining in these structures. In contrast, the vacuolization in the sweat glands showed LAMP2 staining and signs of engulfment of cytoplasmic cargo by late endosomes and lysosomes were also observed. These features are suggestive of microautophagy where cytoplasmic material is directly engulfed by the endosome or lysosome. We hypothesized that microautophagy might play a compensatory role in sweat glands as the three forms of autophagy are complementary to each other.

We excluded known symptoms of lysosomal storage diseases in affected dogs. We did not observe differences in urinary oligosaccharides and glucosaminoglucans between affected and control dogs (Gray et al. 2007). Moreover, measuring the activity of three lysosomal enzymes in cell cultures revealed mildly increased activities in the affected dogs (Warren and Alroy 2000).

Various neurological diseases affect LR dogs, among others, benign familial juvenile epilepsy and cerebellar cortical abiotrophy (Jokinen et al. 2007, Jokinen et al. 2015). Our study described another disease in this breed showing progressive neurological dysfunction characterized by changes in the parathyroid, subcommissural organ and microvasculature. Interestingly, the diagnosis in affected dogs was confirmed, in all cases except one, by the presence of lesions in the sweat glands in skin biopsies. The negative biopsy was from an adult dog homozygous for the ATG4D mutation that presented all the symptoms found in the other affected dogs. A follow-up of this dog would be required in order to understand whether the negative result in sweat glands was due to a lower penetrance of the disease or to reduced sensitivity of the skin biopsies caused by variable distribution of skin lesions between animals.

5.3 Targeting of Beclin 1 and its effects on autophagy

In this study we generated Beclin 1 constructs targeted to the ER and mitochondria in order to study how different subcellular localization of Beclin 1 affects autophagosome biogenesis. Moreover, we also investigated whether forced targeting of Beclin 1 could rescue autophagosome biogenesis in the absence of the ULK1 and ULK2 kinases.

5.3.1 Targeting Beclin 1 to ER or mitochondria does not affect its binding to autophagy-related proteins

To investigate whether forced targeting to ER or mitochondria affects the assembly of the Beclin 1 complex, we used affinity-purification followed by mass spectrometry to detect Beclin 1 binding partners. We showed that all Beclin 1 constructs made abundant interactions with both VPS34 and VPS15. This was an important result, since these kinases are indispensable for autophagosome formation (Petiot et al. 2000, Baskaran et al. 2014, Stjepanovic et al. 2017). UVRAG also made abundant interactions with all Beclin 1 constructs. UVRAG plays an essential dual role in autophagy. UVRAG interaction with Beclin 1 is required for autophagosome maturation (Liang et al. 2008). The absence of UVRAG causes autophagosomes accumulation and severely impairs autophagy flux (Song et al. 2014). Interestingly, a previous study identified an additional role for UVRAG in autophagosome biogenesis (Liang et al. 2006). This study showed that UVRAG can enhance Beclin 1-VPS34 binding thus increasing VPS34 kinase activity and promoting autophagosome formation. We also detected Rubicon as a binding partner for our Beclin 1 constructs. Rubicon negatively regulates autophagy (Matsunaga et al. 2009). Our results showed that Rubicon made abundant interactions with non-targeted Beclin 1 and less abundant interactions with mitochondrially- and ER-targeted Beclin 1, suggesting that the inhibitory functions of Rubicon might be carried out in subcellular locations other than the mitochondria and ER.

We did not detect any Beclin 1 interaction with AMBRA1. AMBRA1 is a known Beclin 1 interactor important for autophagy induction (Fimia et al. 2011, Sun 2016). We hypothesize that the absence of AMBRA1 in our study could be due to its transient interaction with Beclin 1 as AP-MS is most efficient in detecting stable interactions.

5.3.2 Autophagy can occur without ULK1 and ULK2 kinases

It has been shown that in the absence of ULK1 and ULK2 kinases, autophagy is severely impaired (McAlpine et al. 2013). We investigated if Beclin 1 targeting to ER and mitochondria could rescue autophagosome formation in ULK1/ULK2 double knockout MEF cells. ER-targeted Beclin 1 was the most effective in inducing autophagosome formation in ULK1/ULK2 knockout cells while mitochondrially-targeted and wildtype Beclin 1 had similar lower rescue effects.

Several other studies have also shown that autophagosome formation can be induced in the absence of ULK1 and ULK2 kinases. ATG13 is a subunit of the ULK1/ULK2 complex and mediates the interaction between ULK1/ULK2 and FIP200, enhancing ULK1/ULK2 kinase activity (Ganley et al. 2009). One study showed that the absence of ATG13 blocked autophagy induction in DT40 chicken cells (Alers et al. 2011).

ATG13 function was dependent on its binding to FIP200, another component of the ULK complex. However, the simultaneous knockout of ULK1 and ULK2 did not alter autophagy induction in these cells, suggesting a non-essential role for the ULK1/ULK2 kinases in autophagy induction (Alers et al. 2011). Another study investigated non-canonical forms of autophagy regulation (Manzoni et al. 2016). This study showed that autophagosome formation was independent of ULK1 activity but required Beclin 1/VPS34 complex activity (Manzoni et al. 2016). Two further studies also found that autophagy can be induced in the absence of the ULK1/ULK2 kinases (Cheong et al. 2011, Gammoh et al. 2013). More recent research has further shed light on the dispensable role of the ULK1/ULK2 kinases in autophagy induction (Corona Velazquez et al. 2018, Feng et al. 2019). A recent study showed that poliovirus (PV) infection induces autophagosome formation downstream of the ULK1/ULK2 kinases and does not require the canonical autophagic machinery for viral replication (Corona Velazquez et al. 2018). Upon PV infection, autophagy was induced while ULK1 and ULK2 protein levels were significantly reduced, suggesting autophagy induction independent of ULK1/ULK2 kinases. Another study showed that autophagy is robustly induced in early hypoxia (≤ 12 hours 1 % O_2) in MEF cells (Feng et al. 2019). During twelve hours of hypoxia, ULK1 was hyperphosphorylated and remained inactive while LC3-II protein levels increased, again suggesting that ULK1 activity was not needed for autophagosome formation. Interestingly, upon prolonged hypoxia (≥ 24 hours 1 % O_2), the ULK1 complex became active and positively regulated autophagosome formation indicating a fine tuning of ULK1 activity under cellular hypoxic conditions (Feng et al. 2019). All these studies are in line with our findings showing that Beclin 1 can sustain autophagosome formation in the absence of the ULK1 and ULK2 kinases.

5.3.3 Targeting Beclin 1 to ER shows highest efficacy for rescuing autophagosome formation in ULK1/ULK2 deficient cells

Our study revealed that Beclin 1-targeted to the ER showed the highest efficacy for inducing autophagosome formation in ULK1/ULK2 double knockout MEF cells compared to mitochondrially- or non-targeted Beclin 1 in ULK1/ULK2 double knockout MEF cells. This result is in line with previous literature that identifies the ER as the site of autophagosome biogenesis (Hayashi-Nishino et al. 2009, Yla-Anttila et al. 2009, Rubinsztein et al. 2012, Ktistakis 2019). Moreover, our result also supports a pioneering study that showed how autophagosomes emerge from omegasome, an ER subdomain that is enriched with PI3P (Axe et al. 2008) further highlighting the importance of the ER in autophagosome biogenesis.

Mitochondria have also been suggested to deliver membrane material for autophagosome biogenesis (Hailey et al. 2010). Further, ER-mitochondria contact sites have been suggested to contribute to autophagosome biogenesis (Hamasaki et

al. 2013). Owing to these findings, we used mitochondrially-targeted Beclin 1 in addition to ER-targeted Beclin 1 in our studies. However, we detected no difference in the ability of non-targeted Beclin 1 and mitochondrially-targeted Beclin 1 to rescue autophagosome formation in the ULK double knockout cells. Unexpectedly, the mitochondrially-targeted Beclin 1 showed a tendency to support autophagosome maturation, which was not detected with the non-targeted Beclin 1 and ER-targeted Beclin 1. Further work is needed to confirm and explain this finding.

Our findings with ER-targeted Beclin 1 in ULK1/2 knockout MEF cells suggested that Beclin 1 may need to localize in the ER to support autophagosome formation. Indeed, immunofluorescence experiments showed that, upon autophagy induction by starvation, non-targeted Beclin 1 is enriched in the ER. In nutrient-rich conditions, Beclin 1 enrichment in the ER was reduced in ULK1/ULK2 double knockout MEF cells, indicating that ULK1 and ULK2 might regulate Beclin 1 localization under basal conditions. However, under starvation conditions, the ER targeting of Beclin 1 is likely supported by additional mechanisms that are not dependent on ULK1 and ULK2.

5.3.4 Autophagy regulation by modifications of Beclin 1

Beclin 1 itself can regulate autophagy via protein-protein interactions or post-translational modifications. A non-canonical MEK/ERK module (mitogen-activated protein kinase/extracellular signal-regulated kinase) positioned downstream of AMPK, regulates Beclin 1 activity in human erythroleukemia K562 cells and in rat hepatoma H4IIE cells (Wang et al. 2009). This study showed that, upon autophagy induction, AMPK activates the MEK/ERK module which subsequently enhances Beclin 1 activity, modulating autophagy. Another study showed that Beclin 1 interacts with VMP1 (Vacuole Membrane Protein 1) and that this interaction positively regulates autophagosome formation (Molejon et al. 2013).

5.3.5 Role of ULK 1 and ULK2 kinases in autophagosome maturation

Our study showed that while Beclin 1 overexpression was able to rescue autophagosome formation in ULK1/ULK2 double knockout MEF cells, autophagosome maturation was poorly or not at all recovered. Our results are supported by previous findings indicating that ULK1/ULK2 kinases play a role also in autophagosome maturation. Beclin 1 forms two distinct complexes that regulate two different stages of autophagy (Itakura et al. 2008, Liang et al. 2008). The Beclin 1-VPS34 complex assembled with the participation of ATG14 regulates autophagosome formation (Itakura et al. 2008). When the Beclin 1-VPS34 complex contains UVRAG instead of ATG14, it regulates endocytic trafficking and autophagosome maturation (Liang et al. 2008). Upon autophagy induction, the Beclin

1-VPS34 UVRAG-containing complex is phosphorylated by ULK1, and this positively regulates autophagosome maturation (Russell et al. 2013). The absence of the ULK1 and ULK2 kinases causes defective phosphorylation of Beclin 1, which is likely to alter autophagosome maturation. Another study investigated the role of ULK1 in autophagosome maturation and showed that ULK1 directly interacts with STX17 and regulates its binding to SNAP29 which induces autophagosome-lysosome maturation (Wang et al. 2018).

5.3.6 Conclusions on Beclin 1 targeting effects on autophagosome formation

Our study confirms the pivotal role of Beclin 1 in autophagosome biogenesis. We showed that Beclin 1 targeted to the ER was able to sustain autophagosome formation in the absence of the ULK1/ULK2 kinases. In addition, our results support the notion that ULK1/ULK2 kinases are required for autophagosome maturation. Finally, we showed that Beclin 1 is enriched in the ER during autophagy induction, and that ULK1 and ULK2 support ER localization of Beclin 1 under basal conditions.

The main primary method used in this study was the overexpression of Beclin 1. The level of overexpression varies between individual cells and, in the case of strong overexpression, protein folding and localization, stoichiometry of protein complexes and protein-protein interactions can all be affected. In order to overcome this problem, whenever the experimental setting allowed, we selected cells expressing Beclin 1 close to the endogenous level. We were unable to detect endogenous Beclin 1 in immunofluorescence staining. Future experiments could be performed using epitope-tagging of endogenous Beclin 1 with CRISPR/Cas9 technology.

6 CONCLUSIONS

Autophagy is an essential cellular self-eating process that allows cells to degrade intracellular cargoes such as aggregated proteins, whole organelles and foreign bodies. In this doctoral thesis, I have further investigated the molecular mechanisms regulating autophagosome biogenesis in starvation-induced and selective autophagy. TRIM17, ATG4 and Beclin 1 have all been implicated in the pathogenesis of various diseases, and they could be used as potential therapeutic targets.

In our study on selective autophagy, we showed that TRIM17 inhibits autophagic degradation of various cellular targets (Figure 17). TRIM17 interacts with the anti-autophagy protein Mcl-1, which binds and inactivates Beclin 1 sparing cellular targets from degradation. Despite the ability of TRIM17 to inhibit certain forms of selective autophagy, it also positively regulates the autophagic degradation of midbodies. TRIM17-dependent autophagic degradation of midbodies is induced upon dissociation of Mcl-1 from the Beclin 1-TRIM17 complexes formed in connection with the midbodies. Our study elucidated the role of TRIM17 in the process called “precision autophagy” where TRIMs act both as autophagy receptors, by binding directly to the targets, and as platforms facilitating the assembly of core autophagy complexes such as the ULK1 and the Beclin 1 complex. Given its dual role in autophagy, TRIM17, and possibly other TRIMs, add another layer of specificity in the process of selective autophagy by allowing the cell to discriminate or “choose” which cargo to degrade and increasing the specificity of cargo selection.

Our study on basal autophagy in Lagotto Romagnolo dogs revealed that dogs carrying a missense mutation in the ATG4D gene exhibit altered basal autophagy and abnormal cytoplasmic vacuolization (Figure 18). In addition to this, our study also showed that affected dogs present normal lysosomal degradation, excluding a possible link between the ATG4D-gene mutation and lysosomal storage diseases. Our study described a novel condition with distinct phenotypes for yet another progressive neurodegenerative disease in Lagotto Romagnolo dogs, increasing our knowledge on the neurological disorders that affect this breed.

We also showed that in starvation-induced autophagy, Beclin 1 targeted to the ER can partially rescue autophagosome biogenesis in the absence of the ULK 1 and ULK 2 kinases (Figure 19). However, autophagy flux was not rescued by targeting Beclin 1 to the ER. This suggest that ULK1 and ULK2 kinases play a role in autophagosome maturation in addition to autophagosome biogenesis. Moreover, our study also suggests that the ULK kinases might regulate Beclin 1 recruitment to the ER to sustain autophagosome formation. Our study is in line with the primary role of the ER in

autophagosome biogenesis. Further studies are needed to indentify the molecules mediating putative lipid transfer from the ER to the forming autophagosome.

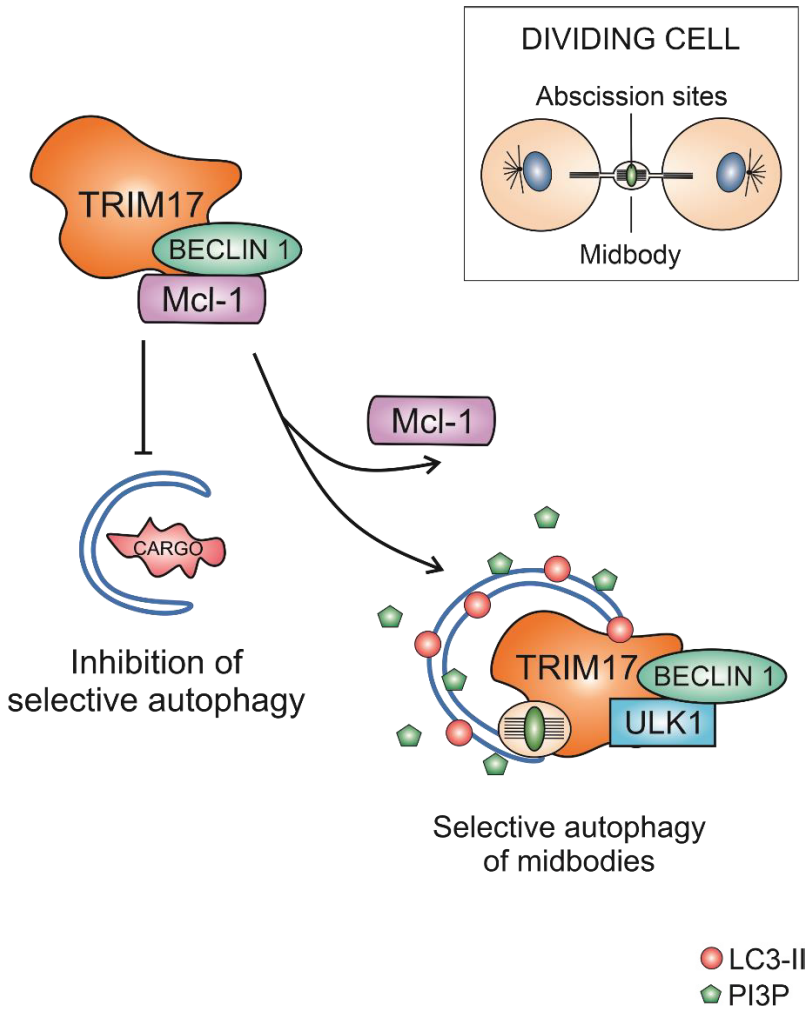

***Figure 17**: Schematic representation of the role of TRIM17 in selective autophagy. TRIM17 acts as a platform and coordinates the formation of the Beclin 1-Mcl-1 complex inhibiting selective autophagy of various cellular cargoes. When Mcl-1 dissociates from Beclin 1, TRIM17 induces selective autophagy of midbodies.*

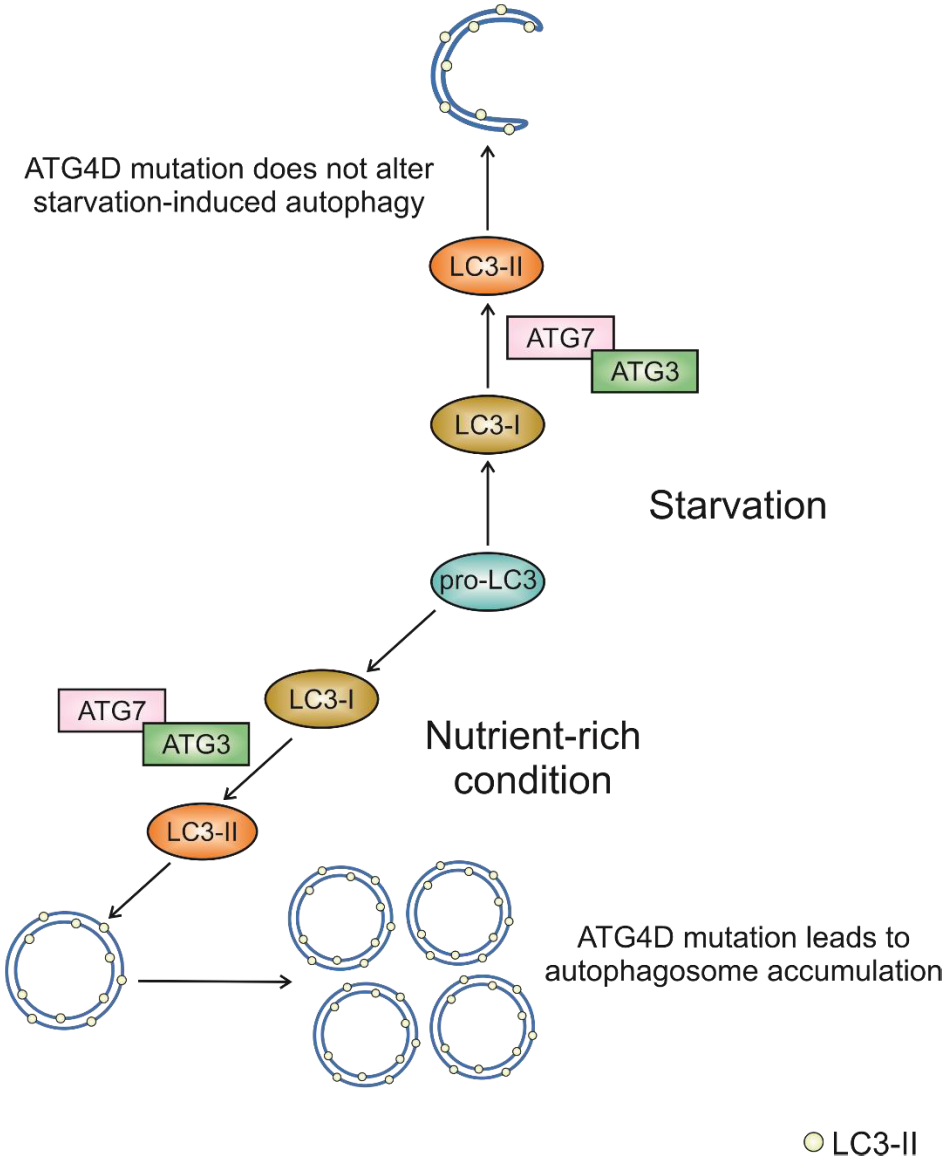

***Figure 18**: Schematic representation of the effects of ATG4D mutation on autophagy. The ATG4D mutation alters basal autophagy while not impairing starvation-induced autophagy.*

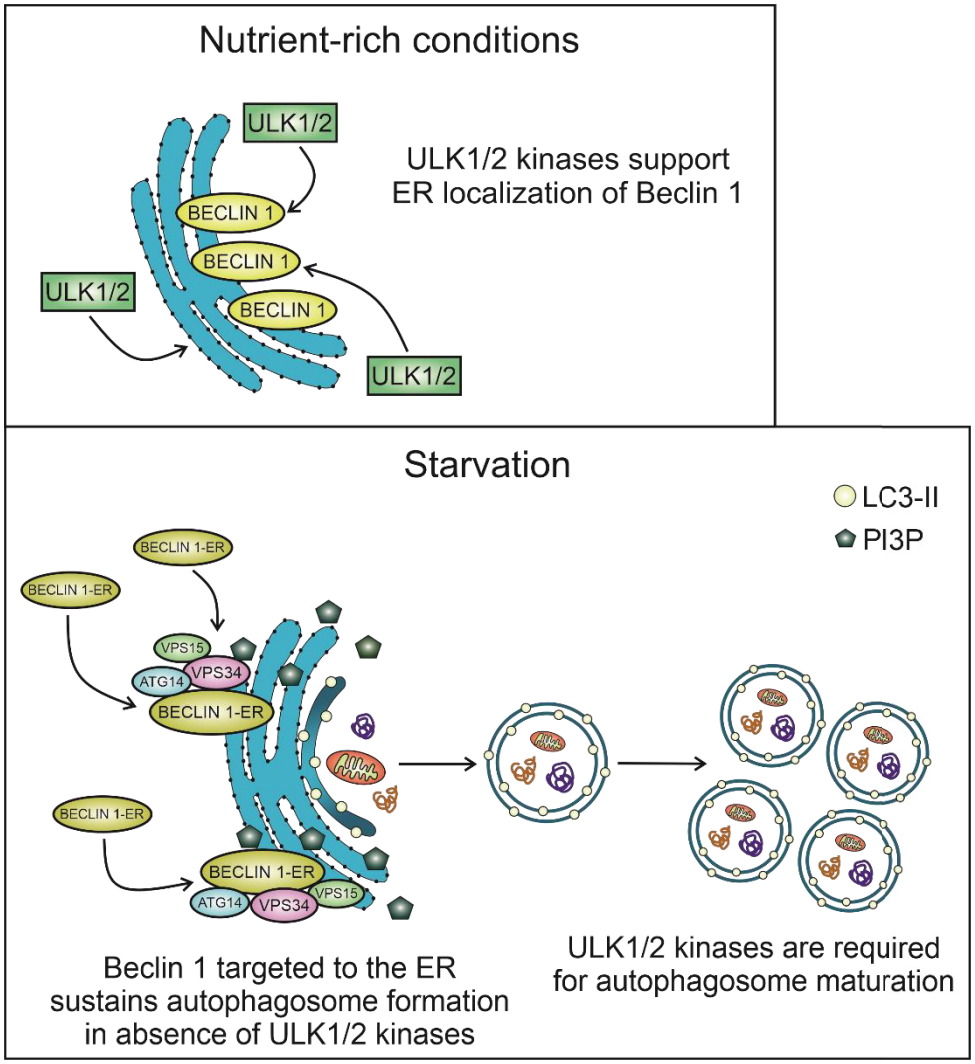

***Figure 18**: Schematic representation of the role of Beclin 1 localization in autophagy. Under nutrient-rich conditions, ULK1 and ULK2 kinases maintain a pool of Beclin 1 in the ER (upper panel). Upon autophagy induction, Beclin 1 is enriched in the ER also in the absence of the ULK1/2 kinases. Autophagy flux is impaired in cells lacking the ULK1/2 kinases suggesting a regulatory role for these kinases also in autophagosome maturation.*

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